PRESENTATION OF AN INTEGRATED CARDIO–NEURO–GENETIC FRAMEWORK IN FETAL MEDICINE

Ph.D. Thesis

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Original publications related to the PhD thesis:

I. Elekes, T.; Csermely, G.; Kádár, K.; Molnár, L.; Keszthelyi, G.; Hozsdora, A.; Vizer, M.; Török, M.; Merkely, P.; Várbíró, S. Learning Curve of First-Trimester Detailed Cardiovascular Ultrasound Screening by Moderately Experienced Obstetricians in 3509 Consecutive Unselected Pregnancies with Fetal Follow-Up. Life 2024, 14, 1632.
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II. Elekes, T.; Ladanyi, A.; Pap, E.; Szabo, J.; Illes, A.; Gullai, N.; Varbiro, S. Second Trimester Ultrasound Diagnosis of External Hydrocephalus in Two Fetuses with Noonan Syndrome-Case Report Series. J. Clin. Med. 2025, 14, 3973.
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III. Pikó, H.; Illés, A.; Nagy, S.; Beke, A.; Árvai, K.; **Elekes, T.**; Horváth, E.; Ferenczy, M.; Mosonyi, P.; Lukács, V.; Klujber, V.; Török, O.; Kiss, Z.; Tardy, E.; Tidrenczel, Z.; Tobiás, B.; Balla, B.; Lakatos, P.; Kósa, J.; Takács, I. Summary of the first Hungarian experiences with prenatal chromosomal microarray analysis and whole-exome sequencing. Orvosi Hetilap 2024, 165(14), 523-530.
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List of abbreviations

AC	Abdominal circumference
ACMG	American College of Medical Genetics and Genomics
ASD	Atrial septal defect
AVSD	Atrioventricular septal defect
BMI	Body mass index
BPD	Biparietal diameter
CAPN3	Calpain 3 gene
CBL	Casitas B-lineage lymphoma proto-oncogene
CC2D2A	Coiled-coil and C2 domain containing 2A gene
CHD	Congenital heart defect
CMA	Chromosomal microarray analysis
CNS	Central nervous system
CNV	Copy number variation
CRL	Crown–rump length
CSF	Cerebrospinal fluid
CVS	Chorionic villus sampling
DNA	Deoxyribonucleic acid
DV	Ductus venosus
EBP	Emopamil binding protein gene
EH	External hydrocephalus
EIF	Echogenic intracardiac focus
FHR	Fetal heart rate
FMF	Fetal Medicine Foundation
GATK	Genome Analysis Toolkit
GJB2	Gap junction protein beta 2 (connexin 26)
HC	Head circumference
HLHS	Hypoplastic left heart syndrome
HRH	Hypoplastic right heart
IVF	In vitro fertilization
KIF21A	Kinesin family member 21A
LCR	Low copy repeat
LV	Left ventricle

MRI	Magnetic resonance imaging
NAHR	Non-allelic homologous recombination
NB	Nasal bone
NGS	Next-generation sequencing
NHEJ	Non-homologous end joining
NIPT	Non-invasive prenatal testing
NS	Noonan syndrome
NT	Nuchal translucency
PFO	Patent foramen ovale
PFSR	Prefrontal Space Ratio
PTPN11	Protein tyrosine phosphatase non-receptor type 11
RASopathy	RAS/MAPK pathway–related syndrome
RV	Right ventricle
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SOS1	Son of sevenless homolog 1
STIC	Spatiotemporal image correlation
SUA	Single umbilical artery
TGA	Transposition of the Great Arteries
TNNT3	Troponin T3, fast skeletal type
TR	Tricuspid regurgitation
US	Ultrasound
VSD	Ventricular septal defect
VUS	Variant of uncertain significance
WES	Whole-exome sequencing

1. Introduction

1.1. Historical overview

Fetal medicine has developed significantly over the past century integrating advances in imaging and genetics to improve perinatal and postnatal outcomes. The development of this field was linked to the progress in biomedical technologies. The history of prenatal diagnostics dates back to the 19th century: in 1896, Edward Parker Davis was one of the first to use X-rays to see the fetus for diagnostic reasons. [1]

By the middle of the 20th century, ultrasound was gaining acceptance in obstetrics. In 1958, the Scottish physician Ian Donald published a paper in The Lancet describing how pulsed ultrasound was used to evaluate abdominal growth. His work established the basis for later prenatal ultrasound examinations. In the early 1960s, further advances were made by scientists such as Robinson, Kossoff, Radovanovich, and Garrett, who demonstrated that the anatomical details of the fetus could be visualized using complex B-mode scanners. [2] This development represented a turning point in the history of prenatal imaging. The introduction of Doppler technology - first pulse Doppler, then color Doppler - made it possible to evaluate fetal heart movement and blood flow in major vessels, making ultrasound the dominant imaging method in obstetrics. [3]

Over the past 20–30 years, fetal echocardiography has benefited immensely from technological innovations. Color Doppler, introduced in the late 1980s, enabled detailed visualization of intracardiac structures and blood flow. [4,5] Subsequent enhancements, including power Doppler, anatomical M-mode, spatiotemporal image correlation (STIC), 3D/4D ultrasound, speckle tracking, and even fetal cardiac MRI, have dramatically expanded diagnostic capabilities and moved anomaly detection earlier in gestation. [5]

The benefits of diagnosing CHD prenatally include earlier genetic counselling, improved understanding of the prognosis, and lower risks – physically and psychologically – for pregnant women. [6–8] Early identification of CHD has the potential to reduce neonatal morbidity and mortality by allowing for planning of intensive postnatal care. [9–13] Pediatric cardiologists use fetal echocardiography to examine high-risk pregnant patients including women with a positive family history of CHD, those who have undergone IVF, those with maternal metabolic disorders, particularly obesity and diabetes, those of advanced maternal age, those with autoimmune diseases (e.g. SLE, Sjögren), fetal teratogenic exposure, and with

an abnormal NT, TR, or DV during genetic ultrasound screening in the first trimester. [14–19] Among this high-risk group of pregnant women, almost all severe CHD cases receive a diagnosis before birth. [20,21] However, the literature records that 90% of CHD cases occur in the low-risk group, for whom routine obstetric ultrasound screening has limited effectiveness in detecting most severe fetal heart abnormalities, as the examination is time-consuming and the examiners mostly lack the appropriate expertise, which could improve significantly after an appropriate learning process. [20–25] Aneuploidy screening performed by obstetric ultrasound specialists with Fetal Medicine Foundation (FMF) certification during the 11–13 week period has improved the first trimester CHD detection rate. [26] Thanks to the widespread use of high-frequency ultrasound scanners, alongside the emergence of new Doppler techniques, means that obstetricians adept in fetal cardiac examination can visualize the four-chamber view and ventricular outflow tracts of the fetus in ~95–97% of cases in the first trimester. [16,27–29] Several international working groups have demonstrated the success (>90% detection rate) of extended fetal cardiac examinations performed in the first trimester by skilled obstetric ultrasonographers in pregnant patients designated high risk. [30,31]

Similarly to the heart scan, fetal neurosonography has advanced from basic 2-dimensional scans to complex 3-dimensional techniques. Advances in morphological assessment have enhanced the detection of central nervous system (CNS) anomalies. CNS malformations occur in approximately 0.3–1% of live births; ultrasonography now detects many CNS anomalies as early as the first or early second trimester, significantly improving clinical preparedness.

Fetal ultrasound has become a key technique in prenatal genetic diagnostics by allowing the measurement of nuchal translucency (NT), a sensitive marker for chromosomal abnormalities such as Down syndrome. In 1990, Prof. János Szabó and János Gellén from Szeged published a landmark article in the Lancet describing that nuchal translucency ≥3 mm in the first trimester is closely associated with trisomy 21. [32] This study significantly contributed to the regional and wider spread of NT-based screening methods. Prof. Kypros Nicolaides played a fundamental role in the development of combined screening in the first trimester, which significantly improved the detection rate of aneuploidies by integrating NT measurement and maternal serum markers. [33–35] With the integration of these techniques, modern prenatal screening protocols have achieved significantly greater accuracy in identifying chromosomal abnormalities in early pregnancy.

The genetic dimension of prenatal diagnostics has developed in parallel with imaging technologies. While the link between Down syndrome and heart defects was already recognized in the 1920s, trisomy 21 was first confirmed cytogenetically in the late 1950s. [36]

Amniocentesis, originally used at the end of the 19th century to remove excess amniotic fluid, became available for diagnostic purposes in the 1970s, mainly for detecting chromosomal abnormalities such as Down syndrome. The development of chorionic villus sampling (CVS) in the 1980s enabled early genetic diagnosis from chorionic tissue. [4]

For decades, traditional cytogenetic testing (karyotyping) of placental and amniotic fluid samples has represented the criterion standard method for prenatal genetic diagnosis, suitable for detecting numerical and gross structural abnormalities in fetal chromosomes. This method can detect numerical abnormalities (such as trisomies and monosomies) and certain structural abnormalities (such as breaks, translocations, duplications, and deletions). The resolution limit of the currently used, so-called traditional chromosome analysis is approximately 10–12 Mb. Smaller abnormalities cannot be detected using classic cytogenetic methods based on light microscopy. [37]

As a result of abnormalities in the smaller range affecting multiple genes, which cannot be detected by conventional cytogenetic testing, complex and severe symptom complexes may develop, which are referred to as microdeletion/microduplication syndromes. These syndromes are usually severe conditions that can be associated with intellectual disability, neurological symptoms, and complex organ disorders. Chromosomal microarray analysis (CMA) provides a method for identifying small deletions/duplications (microdeletions and microduplications, or copy number variations [CNVs]). [38,39]

Since 2010, next-generation sequencing (NGS) has made tremendous progress in identifying and understanding variations affecting a few nucleotides in relation to various diseases using whole-exome sequencing (WES). This high-throughput next-generation method has become an indispensable part of prenatal diagnostics thanks to falling costs and advances in bioinformatics algorithms. [40] The positive added value of WES testing is around 8–10% after negative CMA and karyotyping results.

In the last decades, non-invasive prenatal testing (NIPT), using cell-free fetal DNA in the circulation of the mother, has revolutionized the screening process, with a detection rate for trisomy 21 exceeding 99% and a false positive rate of approximately 0.1%. [4] Meanwhile,

chromosomal microarray analysis (CMA), exome sequencing, and next-generation sequencing have progressively improved the diagnostic performance for subchromosomal or complex syndromic conditions. [7,36]

1.2. Main epidemiological data

The precise number of all structural and genetic abnormalities present during fetal life is difficult to determine, as the availability of routine screening, the depth of the examinations performed, and the detection rate vary by geographic region and even by institution (or by examiner within the same institution). Furthermore, even in postnatal life, the correct diagnosis is not always made quickly (and in some cases not at all). According to the currently available estimates, approximately 3-6% of fetuses in the general population have a structural anomaly or a genetic disorder, often in association with each other. [41–45]

The most common major congenital anomaly is congenital heart disease (CHD). About half of the CHD cases are minor and, if necessary, can be corrected by surgery, while the other half account for more than half of all childhood deaths from congenital anomalies. The incidence of CHD depends on the definition of CHD as well. Furthermore, when considering subtle abnormalities such as persistent superior vena cava and isolated atrial septal aneurysm, the total incidence of CHD approaches 50/1000 live births. [46] According to EUROCAT's aggregate data, between 2005 and 2023, the total prevalence of "all anomalies" was 265.24 per 10 000 births, while congenital heart defects (CHD) were 81.54 per 10 000, accounting for approximately 31% of those born with a structural anomaly. [47] The recent ISUOG guideline on fetal heart screening reported that the prevalence of CHD is approximately 8.2/1000 live births. [48] Given that the detection rate is estimated to be approximately 40-60% for heart defects, the actual prevalence in the fetal period could be up to 1.5-2% for all heart defects.

Similarly, it is also challenging to obtain accurate data on the anomalies affecting the central nervous system (CNS). It is also likely that there are significant differences in the thoroughness of routine examinations and detection rates for the CNS, and even the postnatal diagnosis may be delayed. ISUOG suggests that up to 1% of all fetuses could be affected, but perinatal data provides estimates well below this level. [49] EUROCAT lists 27.35 per 10,000 births for “nervous system anomalies”, which relative to their overall anomaly rate corresponds to ~10% of all congenital defects.

The exact prevalence of genetic abnormalities can also be only estimated due to the reasons previously mentioned and the fact that they often do not show obvious signs. According to some estimates, up to over 5% of the normal population might be affected by a genetic condition of varying severity. Genetic disorders - defined by EUROCAT to include chromosomal anomalies and genetic syndromes - show a prevalence of 58.05 per 10,000 births, or ~22% of all congenital anomalies. [47]

Overall, it can be said that a significant proportion of all fetuses with developmental abnormalities show cardiac or central nervous system anomalies, which are often associated with genetic syndromes.

The proportion of genetic disorders detected by different diagnostic tools shows a significant variability in the current available literature. Depending on the severity and number of fetal anomalies, approximately 30% of structural defects detected by ultrasonography can be diagnosed as aneuploidy. When analyzed sub-chromosomally (CMA), additional 4-15% of genetic abnormalities can be detected after a negative karyotype. The further yield (in addition to negative karyotype + CMA) of exome sequencing (WES), which examines the protein-coding regions only (1-2% of the total DNA), varies between 8-30%. [50–54] Even with the use of all three techniques, a significant proportion of fetuses with structural abnormalities remain without an adequate genetic diagnosis.

The ability to perform in-depth cardiovascular and neurosonographic examinations of the fetus is, among others, an important prerequisite for the precise planning of genetic testing (NIPT, karyotiping, CMA, WES) and other complementary tests. The high-level competence of future clinical geneticists and ultrasound examiners in fetal echocardiography and neurosonography will not only be able to detect the vast majority of fetal structural abnormalities but will also provide a favorable effect on the ability to utilize the narrowed genetic decision-making window due to the time-consuming nature of molecular testing.

1.3. Legal and social context

In different countries, legislation concerning the protection of fetal life is formulated in different ways, depending on the cultural, religious, ethical, and societal characteristics of each country. In Hungary, based on genetic indications, couples can decide up to the 23rd week and 6th day of pregnancy to terminate the pregnancy if the probability of genetic or severe structural

damage to the fetus reaches 50%.[1] The fetal diagnostic procedures may begin in the 9-10th week of pregnancy and must be completed by the 24th week so that the couple can make an informed decision about the future of the pregnancy. Therefore, the accuracy of diagnostic procedures and shortening the time between the initial suspicion and the final diagnosis are of crucial importance.

Beyond legal considerations, decisions about pregnancy are also influenced by the cultural and religious traditions of a given country or region, the strength of personal and community support, and the quality of health and social services available in the country.

[1] See the Appendix for the complete text of the relevant law.

2. Aims of this thesis

As outlined in the introduction, prenatal diagnostics has evolved significantly over the past two decades. Given this rapid development, a key clinical question is how to design and implement the most accurate and effective screening algorithm for pregnant patients — one that can either provide reassurance or timely, high-quality information to support decisions regarding the continuation of pregnancy, within the time limits set by law.

According to the author's opinion, the most reliable method for characterizing fetal abnormalities and predicting their prognosis is the simultaneous, systematic examination of the fetal heart and central nervous system, combined with genetic evaluation — an approach that can be implemented within a ‘cardio-neuro-genetic’ framework. Such an approach allows for precise planning of the diagnostic process and improves the quality and consistency of genetic counseling and decision-making. This approach requires early, extended ultrasound screening, which has strict technical and human resource criteria.

A frequently raised question concerning extended early screening is whether a general obstetrician-gynecologist is capable of acquiring the skills necessary for such detailed examinations, and if so, how long the learning period would take, and whether these extended examinations are even justified in routine practice. Another commonly raised question is what practical benefit does the routinely performed detailed mid-trimester neurosonography have. The third question that comes up regularly is what our experience with the performance of subchromosomal genetic testing in Hungary is.

This thesis aims to address these questions by studying the following topics:

- To evaluate the learning curve of obstetric and gynecologic specialists in performing detailed early fetal echocardiography.
- To demonstrate, through selected cases, the clinical value of routinely performed detailed neurosonography.
- To present our experience with a staged, three-level genetic diagnostic approach (karyotyping – microarray – WES) in Hungary.

3. Materials and methods

3.1. Examination of the learning curve of obstetrician-gynecologists performing first trimester fetal cardiac screening

A retrospective study was conducted to analyze the learning curve of obstetrician-gynecologists performing first trimester fetal heart scans. The study received approval from the Hungarian Medical Council Scientific and Research Ethics Committee (2013/EKU - 588/2013).

The learning curve was analyzed using data from fetal heart scans performed on our general pregnant population during the first trimester at the RMC-Fetal Medicine Center between 1 January 2010 and 31 January 2015.

The early fetal echocardiographies were conducted alongside the standard first-trimester extended ultrasound screening. This also involved a full anatomical evaluation of the fetus with observation for signs of aneuploidy, together with preeclampsia screening according to the FMF guidelines. Transabdominal ultrasound was performed using Accuvix V20 (Samsung-Medison, Seoul, Republic of Korea) and Voluson S8 (GE Medical Systems, Florence, SC, USA) ultrasound machines fitted with a 4–8 MHz convex abdominal transducer.

At the start of the study duration, ultrasound scans were conducted by three obstetricians (ultrasound specialists for NT, NB, DV, and TR, audited by the FMF) who, while familiar with the normal first and second trimester fetal anatomy, had only moderate experience evaluating fetal heart abnormalities. In the final year of the study period, an FMF-audited ultrasound technician joined the team to prescreen the patients.

For all patients, a full medical history was obtained, which included data on the mothers' height, weight, blood pressure, and temperature.

A new institutional standardized protocol was applied for first trimester fetal echocardiography from the 1st of January 2011. Accordingly, clinicians were required to examine and digitally archive the following: determination of abdominal situs; size, position and axis of the heart, 4-chamber view: crux cordis, ventricular septum in the four-chamber view, ventricular filling, tricuspid pulsed wave Doppler, and FHR; 3-vessel view, V sign; longitudinal view: aortic arch with color flow, ductal arch, right ventricular outflow tract,

transposition of the great arteries; and optional examination of the left atrial pulmonary vein (minimum two) and the right atrium - vena cava connections (for venoatrial concordance).

Cardiac screening using this protocol was held as comprehensive provided all the above-mentioned elements were stored digitally. The examinations were not subject to any time limits, and were terminated only if there were other obstacles to visualization besides the position of the fetus; for example, where the transducer was too far from the fetus (variously due to retroflected uterus, thick maternal abdominal wall, previous abdominal scars, or anterior placenta), and visualization could neither be achieved via vaginal ultrasound.

In normal cases during the first trimester, where the patient returned, the cardiac screening was repeated at weeks 18–20 and 28–30. Any routinely screened cases considered "abnormal", together with any high-risk cases, were re-examined by a pediatric cardiologist. Parents were given full genetic counseling if prenatal cardiac screening revealed any abnormality.

Those pregnant women who had screened positive for aneuploidy in the first trimester, and those indicated by the type of CHD, were offered fetal karyotyping by chorionic villus sampling or an amniocentesis test. Women screened at our center had received routine prenatal, genetic, and general obstetric care at a number of institutions nationwide (221 attending physicians), so we collected data on patient pregnancy outcomes using three methods: patient response to email questionnaire (our email address was included in the report following the first trimester obstetric ultrasound); data obtained from digital records held by our center's obstetrics department, where patients received prenatal care; verbal interviews conducted by telephone between medical staff and the mothers. If the results were unclear, the obstetrician conducted a repeat interview on the phone to obtain clear information.

For statistical analysis, a chi-square test was performed, for which a value of $p < 0.05$ was considered to be a significant difference. GraphPad Prism 6.0 software was used to evaluate data.

3.2. Examination of prenatal chromosomal microarray analysis and whole-exome sequencing in Hungary, based on our experience

Based on our former experiences, at the start of the study our working group has developed a diagnostic algorithm in accordance with international standards, according to which, when prenatal genetic testing is indicated, i) we first perform traditional karyotyping, and if the result is negative, ii) we proceed to CMA with higher resolution, and if this is also negative, iii) we continue the diagnostic series with WES testing, which can identify single nucleotide variations.

CMA tests were performed using the GeneChip System 3000 Instrument (Affymetrix, Santa Clara, CA, USA) platform: the GeneChip Hybridization Oven 645 (Thermo Fisher Scientific, Waltham, MA, USA) hybridization chamber was used for hybridization, the Gene-Chip Fluidics Station 450 (Thermo Fisher Scientific) device was used for washing, and the Gene-Chip Fluidics Station 450 (Thermo Fisher Scientific) device was used for measuring signal intensity.

The test is based on an SNP-based comparative genomic hybridization method. The DNA sample is digested with NspI enzyme into pieces of different sizes, then an adapter molecule is attached to them, with which whole genome amplification is performed. The amplified sample is labeled (with biotin) and then hybridized to 25-nucleotide oligonucleotide probes on the solid phase of the array, which cover the entire human genome. Next, during the washing step, we remove unbound DNA fragments and other contaminants from the solid phase, and stain the biotin-labeled DNA fragments bound to the probes with a streptavidin-phycoerythrin complex. This creates a biotin-streptavidin-phycoerythrin complex that can be excited and emits in the 572 nm range. When measuring signal intensity, the device detects the emitted signal according to the copy number ratio, and the final analysis allows us to visualize the individual chromosomes, in which the system also marks any deficiencies or surpluses.

During the NGS we performed, we determined the complete human exome sequence in 42 prenatal samples. Sequencing was performed on IonTorrent (Thermo Fisher Scientific) and Illumina (Illumina, San Diego, MA, USA) platforms. After extracting genomic DNA of sufficient quality and quantity, we amplified the entire human exome as the first step in library preparation, then, after several purification and labeling steps that enabled multiplex parallel sequencing, we checked the concentration of the finished libraries.

Next, the libraries were loaded onto the sequencing platform (IonChip or FlowCell) in equimolar amounts. After sequencing, we aligned the resulting sequences to the reference genome (hg37) and then performed variant calling using GATK v4.1.4.1 software. We used Franklin by Genoox to annotate and interpret the variants found.

In all cases, patients signed a consent form for the genetic testing, which is stored by the relevant institutional unit for the period specified by the law, and the data were processed retrospectively in an anonymized manner.

3.3. Presentation of the importance of mid-trimester detailed neurosonography through two cases

Presented here are two cases of Noonan syndrome in which external hydrocephalus developed in the second trimester alongside variations in the *SOS1* and *PTPN11* genes.

According to the available literature, this is the first reported association to date between prenatal external hydrocephalus in Noonan syndrome and variation in the SOS1 gene. Additionally, these cases represent the first reported ultrasound diagnoses in the second trimester in NS. CARE guidelines were used for structuring the study. External hydrocephalus was determined at the level of the Sylvian fissure: values above the 95th percentile were defined as EH.

4. Results

4.1. Our results from the study of the learning curve for first-trimester fetal heart screening performed by obstetricians and gynecologists

Altogether, 42 (0.88%) congenital heart defects were detected among 4,769 fetuses (4,441 singletons, 155 twins, and 6 triplets) whose mothers received first-trimester cardiac ultrasound screening from 1 January 2010 to 31 January 2015. Table 1 summarizes the distribution of different types of CHD by year of detection.

Table 1. Prenatally observed abnormal hearts during the learning curve.

	2010	2011	2012	2012	2014	Total
# of 1st-trim. examinations	228	586	872	1228	1855	4769
# of abnormal hearts (1st-trim. Dx)	2 (1)	8 (7)	6 (5)	15 (14)	11 (9)	42 (36)
HLHS		2		1	1	4
AVSD	1	1	1	1	3	7
AVSD + HRH		1				1
AVSD + HLH			1		1	2
AVSD + Heterotaxia		1				1
VSD		1	2	3	1	7
VSD + LV < RV				1		1
ASD + LV < RV			1			1
LV > RV					1	1
LV < RV					1	1
HLH + VSD				1		1
HRH + VSD		1				1
Aortic stenosis + HLH + AVSD				1		1
Aortic atresia	1	1				2
Pulmonary valve regurgitation					1	1
Pulmonary atresia + HRH				1		1
Septal fibrosis + VSD				1		1
Ventricular fibro-elastosis				1		1
Rhabdomyoma					1	1
TGA					1	1
Tetralogy of Fallot				1		1
Isolated pericardial fluid			1	1		2
CHD—non evaluated				2		2

Abbreviations: number , number; Dx, diagnosis; HLHS, hypoplastic left heart syndrome; AVSD, atrio-ventricular septal defect; HRH, hypoplastic right heart; HLH, hypoplastic left heart (LV << RH); VSD, ventricular septal defect; LV, left ventricle; RV, right ventricle; ASD, atrial septal defect; TGA, transposition of great arteries; CHD, congenital heart defect.

Due to the small number of cases recorded in the initial year of the study (2010, n = 228) and the lack of an examination protocol for fetal heart evaluation, fetuses screened in this period were excluded from the study. Among those screened between June and December 2014 (n = 1032 fetuses), most were still prenatal during analysis of results (between 1 February 2015, and 31 March 2015). Thus, fetuses screened between 1 January 2011 and 30 June 2014 were followed up, and outcome data for these fetuses allowed evaluation of screening effectiveness.

Over the course of this 42-month period, 3,372 pregnant women carrying 3,509 fetuses (125 twins, 6 triplets, and 3,241 singletons) underwent full fetal cardiac ultrasound screening at 11+0 – 13+6 weeks of gestation. There was a follow-up rate was 93% (3,142/3,372). Of the 239 fetuses from 230 pregnancies that were not followed up, no abnormalities were identified at the first-trimester scan.

Accordingly, 3270 fetuses were tracked, comprising 3020 singletons, 116 twins, and 6 triplets. The average age of the mothers at first-trimester screening was 33.9 years (17–45). The proportion of pregnant women over 35 years of age was 46.9%. The average crown-rump length (CRL) of the fetuses was 64.0 mm (45.0–84.0). At first-trimester screening, the average maternal age in the total study population was 33.9 years, while the average age in the pregnancy group with severe fetal CHD was 35.8 years (not significant, $\chi2 = 3.601$, df = 1, p = 0.058). The respective proportion of women over 35 years of age in the total study population and in the pregnancy group with severe fetal CHD was 46.9% and 71.0% (significant, $\chi2 = 6.642$, df = 1, p = 0.0099).

NT, DV, and TR measurements (FMF software version 2.3) conducted simultaneously with fetal heart screening showed 56.1% of NT values to be above the median, while 4.9% were above the 95th percentile. However, in 54% of heart defects detected prenatally (20/37), NT values were beneath the 99th percentile (<3.5 mm), while in 46% of such cases (17/37), the NT was below 2.5 mm. Regarding other cardiac markers, TR was identified in 0.6% of non-CHD instances and in 29% of CHD fetuses (10/35) (significant, $\chi2 = 308.486$, df = 1, $p < 0.05$). Correspondingly, abnormal DV blood flow patterns were found in 4.3% of all fetuses screened and in 51% of fetuses shown to have cardiac abnormalities (18/35) (significant, $\chi2 = 168.282$, df = 1, $p < 0.05$).

4.1.1. Prenatal diagnosis

At the first-trimester heart screening of 3,270 fetuses, moderately experienced obstetricians identified 34 hearts as "abnormal". Of these, two cases were considered normal (number 33 and 34), in five cases (number 4, 14, 21, 24, and 32) the first diagnosis was modified or appended, and three cases (number 6, 15, and 18) were not examined by a pediatric cardiologist. In 24 cases, the cardiologist concurred with the first diagnosis. In those three cases that were not reviewed by a pediatric cardiologist, the patients did not opt for the fetal echocardiograms due to other associated fetal congenital anomalies, and instead chose to

terminate the pregnancy. Of the 32 prenatally confirmed fetal heart defects (which were examined by practiced obstetricians and pediatric cardiologists), 6 were classified as "minor defects" (number 3, 10, 28, 30, 31, and 32), with the remaining cases (n = 26) classified as " major heart defects".

Table 2. Abnormal cardiac findings during the following period.

	1st-Trim. OB. Dx	1st-Trim. Card. Dx	NT	TR	Abn. DV	20-Week OB + Card.	Assoc. Malformations	Karyotype	Pregnancy Outcome
1	AVSD	idem	2.3	+	+	-		47XX, +21	Termination
2	AVSD, Heterotax-iaDextrocardia	idem	1.7	not done	+	-	-	-	Termination
3	VSD	idem	7.9	+	+	-	-	-	Termination
4	AVSD + HRH	id. + fibro-elast	2.5	-	+	-	SUA	46XY	Termination
5	AVSD + HLHS	idem	1.9	-	-	-	-	46XX, 16 polymorph.	Termination
6	LV < RV, ASD	Not ex-amined	5.1	-	-	-	Omphalocele paklatoschisis	47XX, +13	Termination
7	Tricu. atr, HRH, VSD	idem	1.6	Flow:-	-	-	-	46XX (abortum)	Termination
8	AVSD	idem	5	+	_	-	Oligohydramnios	-	Termination
9	HLHS	idem	6	+	-	-	Holoprosencephalia Polydactylia	47XY, +13	Termination
10	VSD (subaortic)	idem	N/A	not done	not done	-	Hydronephrosis Camptodactylia SUA oligohydramnion	69XXX	Termination
11	VSD, fibro-leastosis	idem	2.1	-	-	-	-	-	Termination
12	PA atr., HRH	idem	3.9	-	-	-	-	-	Termination
13	RV fibro-elastosis	idem	5.5	not done	not done	-	-	-	Termination
14	HLHS, VSD	HLHS, AVSD Aorta stenosis	1.5	+	not done	-	-	-	Termination
15	Left rot., Abn. GA	Not ex-amined	6	not done	+	-	cervical cyst, short bones	PCR negative Cytogenetics cannot be performed:	Termination
16	HLHS	idem	2.4	-	+	-	Alob. holoprosencephaly Palatoschisis SUA	-	Termination
17	HLHS, VSD,	idem	7	-	not done	-	Holoprosencephaly Encephalocele Omphalocele	-	Termination
18	Abn. 4-CV + outfl. tr.	Not ex-amined	8	not done	+	-	-	-	Termination
19	LV < RV, VSD	idem	7.3	-	-	-	Cleft lip and palate	47XX, +13	Termination
20	Tetralogy of Fallot	idem	4.5	.	+	-	-	-	Termination
21	LV < RV, AVSD	only AVSD	3.6	-	+	-	-	47XX, +21	Termination
22	AVSD	idem	4.7	+	+	-	Clubfoot, short bones	-	Termination
23	LV < RV,	idem	9	not done	+	-	Strawberry shape skull	-	Termination
24	RV > LV, P. valve reg.	P.valve reg.	1.8	+	+	-	-	47XX, +17 Chr 16 polymorph.	Termination
25	AVSD	idem	5.1	+	+	-	Short bones	-	Termination
26	AVSD	idem	6.5	+	+	-	-	-	Termination
27	HLHS	idem	1.2	not done	+	-	Omphalocele Cheilo-palatoschisis	-	Termination
28	Septal fibrotic area, Rhabdomyoma	idem	1.6	-	-	idem + VSD; Swiss cheese VSD	-	46XX	Born, idem, closed VSD No VSD Ventricular septum in upper third of echo-dense terime

	1st-Trim. OB. Dx	1st-Trim. Card. Dx	NT	TR	Abn. DV	20-Week OB + Card.	Assoc. Malformations	Karyotype	Pregnancy Outcome
29	HLHS	idem	3.1	+	+	-	-	47XX, +21	Termination
30	Isol. pericard. fluid	idem	2.1	-	-	hasn't come back	-	-	Born, complex P. atresia 1 year old with com-plex pulmonary atresia
31	Isol. pericard. fluid	idem	1.8	-	-	normal heart	-	-	Born, healthy
32	Large VSD	Min. inlet VSD	2.8	-	-	normal heart	-	46XX	Born, healthy
33	RV < LV	normal heart	4.8	+	-	normal heart		46XX, NT panel negative	Born, healthy
34	PA < Ao	normal heart	2.1	-	-	normal heart		-	Born, healthy
35	normal heart	-	2.4	-	-	VSD (subaortic)	Cleft lip	46XY	Born, VSD closed
36	normal heart	-	2.4	-	+	Ao. atr, LV > RV, fibr endocardialis fibrosis		46XY	Termination
37	normal heart	-	2	-	-	3-trim. Rhab-domyoma; Multiplex rhabdomy-oma	-	-	Born, Dx proved
38	normal heart	-	1.9	-	+	VSD (inlet)		46XY	Born, Dx proved
39	normal heart	-	3.3	-	-	small VSD	-	NIPT: negative	Born, cl. lip, VSD closed

Abbreviations: HLHS, hypoplastic left heart syndrome; AVSD, atrio-ventricular septal defect; HRH, hypoplastic right heart; HLH, hypoplastic left heart (LV << RH); VSD, ventricular septal defect; LV, left ventricle; RV, right ventricle; ASD, atrial septal defect; TGA, transposition of great arteries; CHD, congenital heart defect; Dx, diagnosis; OB, medium-experienced obstetrician; Card, pediatric cardiologist specialist; NT, nuchal translucency; TR, tricuspidal regurgitation, DV, ductus venosus; Abn, abnormal; Assoc, associated; Tricu atr, tricuspidal atresia; PA, pulmonary artery; Rot, rotation; GA, great arteries; 4-CV, four-chamber view; Outfl. tr., Outflow tracts; P. valve reg., Pulmonary valve regurgitation; Pericardial, pericardial; Ao, Aorta; idem (id), identical; N/A, non-available; SUA, single umbilical artery; Alob, alobar; cl. lip, cleft lip.

CHD was classed as a "major defect" where heart surgery was necessitated within 12 months postnatally. Two minor abnormalities identified by the pediatric cardiologist during the first trimester examination (number 31: isolated pericardial effusion; and number 32: minor ventricular septal defect, VSD) were judged to be "normal" during the second trimester scan. In one case, where both the obstetrician and the cardiologist had noted an area of fibrosis in the fetal septum at the first trimester cardiac screening, a VSD was revealed at the second trimester scan (number 28). Case 30 (isolated pericardial fluid detected during first trimester screening), which was initially considered to be a "minor abnormality," revealed itself as a major heart defect after delivery (complex pulmonary atresia, Table 2).

Among the 3,238 first-trimester fetal hearts classified as "normal" by obstetricians with a moderate level of experience, abnormal NT, DV or TR detected in the second trimester screening prompted echocardiography, which revealed three additional minor abnormalities (VSD; cases 35, 38, and 39) and one severe abnormality (case 36: aortic stenosis + diffuse

endocardial fibroelastosis). A positive family history in one case prompted intensive fetal cardiological monitoring and follow-up, but no abnormalities were detected in either the first or the second trimester screening. However, multiple rhabdomyomas were detected in the third trimester (case 37).

4.1.2. Pregnancy Outcomes

Among the 3,270 fetuses tracked, 3,191 (97.6%) survived birth. A total of 79 fetuses (2.4%) were stillborn or died soon after birth, and perinatal mortality occurred in 8 cases due to infection or indeterminate causes (Table 3.). No severe CHD was observed within this group. In 18 cases (0.5%), spontaneous abortion occurred in the second trimester; no major CHD was observed in this group, and cardiac screening in the first trimester was negative in all cases. Finally, 53 pregnancies (1.6%) were terminated on the basis of genetic issues, of which 29 were diagnosed with CHD.

Table 3. Outcome of pregnancy in the follow-up period.

Number of Pregnancies Followed	**n = 3270**
Live births	3191 (97.6%)
Pregnancy losses	79 (2.4%)
Perinatal deaths (intrauterine demise + neonatal death)	8 (0.2%)

4.1.3. Postnatal Diagnoses

Altogether, 13 minor heart defects were diagnosed in 3,191 newborns (10 cases of benign heart murmurs and 2 cases of PFO were not included in the analysis because these conditions are not classed as heart defects, Table 4). In the 30th case, a pediatric cardiology examination of an infant at one year of age resulted in a diagnosis of a major heart defect (complex pulmonary atresia, Table 2.). The cardiac examination performed in the first trimester had not reported any abnormalities in the four-chamber view and ventricular outflow tracts, but isolated pericardial fluid was reported. A fetal echocardiography was scheduled; however, the expectant mother did not present for this or the routine second- and third-trimester ultrasound examinations.

In case 32 (minor VSD), a fetal echocardiography performed in the second trimester revealed no evidence of the minor cardiac abnormalities suspected during first-trimester screening, which was confirmed by normal neonatal cardiac function. In case 28, a fetal echocardiogram performed in the second trimester confirmed the diagnosis of septal fibrosis

with VSD suspected in the first trimester, a finding that was only partially confirmed postnatally (the VSD was later found to have closed). In case 39, the first cardiac examination in the first trimester showed a normal fetal heart, with the NT 3.3 mm and the non-invasive prenatal test (NIPT) result normal. However, fetal echocardiography in the second trimester revealed a small VSD, which after birth was found to have closed. The remaining two diagnoses of minor fetal heart defects determined by fetal echocardiography in the second trimester were confirmed by postnatal cardiac examination. Overall, cardiac defects were reported in 49 (1.49%) of the 3,270 fetuses tracked.

Table 4. Postnatally recognized cardiac findings.

Type of Cardiac Anomaly	Number of Fetuses
Ventricular septal defect (2–5 mm)	8
Atrial septal defect II	3
Aortic valve stenosis min. grade	1
Bicuspidal aortic valve + PFO	1
Complex pulmonary atresia	1
TOTAL	14
(PFO + innocent cardiac murmurs)	(2 + 10)

Abbreviation: PFO: patent foramen ovale.

There were fourteen heart defects (28.6%) detected for the first time only after birth (1 severe defect, 13 minor), with the remaining 35 of the 49 heart defects (71.4%) diagnosed before birth (27 severe defects and 8 minor). Notably, only two of the four prenatally detected small VSDs, were confirmed after delivery, suggesting spontaneous intrauterine closure in minor fetal heart septal defects. Among the followed fetuses, 96.4% (27 out of 28) of severe CHDs received prenatal diagnosis by mid-level obstetricians working alongside pediatric cardiologists, while 89.2% (25 out of 28) received either a diagnosis or a reported indication via the first-trimester screening and following fetal echocardiography.

During year one of the five-year study duration (2010), the study goal was to assemble a large enough archive of high-quality images of the fetal heart in the first trimester; accordingly, study reports comprise only the true four-chamber view rather than the full cardiological examination panel. Among the 282 cardiac examinations performed in the first trimester, there was in the second trimester just one case of abnormal fetal heart with atrioventricular septal defect (AVSD), along with a single case of aortic stenosis (Table 1). In the 2011–2012 (follow-up) study period, a total of 11 severe heart defects were reported within a notably larger study group (n = 1458), 9 of which were detected during the first trimester

scan. Regarding the two instances in which no CHD was detected, in the first (case 36, aortic atresia, left ventricle >> right ventricle, fibrosis, refer to Table 2), it was reported that the four-chamber view in the first trimester appeared normal (the aorta appeared slightly narrower in subsequent offline analysis) and the patient had a high risk of trisomy 21 (1:4); which was confirmed negative through chorionic villus sampling (CVS). A conclusive diagnosis of fetal aortic atresia was made during the 18-week scan. The other undiagnosed heart defect was only identified postnatally (complex pulmonary atresia). Among the 9 severe heart defects identified in the first trimester, there were two cases where detailed fetal echocardiography enabled diagnosis of Down syndrome (case 1: AVSD and case 29: hypoplastic left heart syndrome, HLHS). In cases 2, 4, 5, and 7, fetal heart defects were detected alongside normal NT values, and the karyotype was also normal. There was one case (case 2) where, based on the evaluation of the situs and four-chamber view, the final diagnosis was a combination of AVSD and heterotaxy (Table 2). In the latter half of the study duration (2013–2014), all severe heart defects were identified during the first-trimester screening, except for a single case of fetal rhabdomyoma (Table 1).

4.2. The first Hungarian experiences with prenatal chromosomal microarray analysis and whole-exome sequencing

A total of 252 prenatal cytogenetic tests were performed. In 92.8% of cases, the indication for CMA testing was some type of structural fetal ultrasound abnormality (Table 5).

Table 5. Indications for our CMA and WES examinations (252 cases).

Indication	Cases (n)	%
Fetal structural ultrasound abnormality (see Table 2)	234	92.8
Positive NIPT result	2	0.8
Positive family history	4	1.6
CNV confirmed by karyotyping (breakpoint determination)	2	0.8
Marker chromosome confirmed by karyotyping	2	0.8
Balanced translocation in parents	3	1.2
Spontaneous miscarriage (examination of abortus)	3	1.2
Stillbirth (after 24th week)	2	0.8
Total	252	100

Abbreviations: CMA = chromosomal microarray analysis; CNV = copy number variation; NIPT = non-invasive prenatal testing; WES = whole exome sequencing.

The indication for WES testing was generally a negative karyotype and CMA, or an ultrasound abnormality suggesting a genetic cause. The most common ultrasound anomalies

were fetal hydrops, cystic hygroma, and thick nuchal fold (40.60%), craniospinal and craniofacial abnormalities (20.51%), and cardiovascular abnormalities (17.95%) (Table 6). Of the fetal samples examined, 147 (58%) showed no abnormalities using the CMA method with the Affymetrics Optima medium-resolution array.

Table 6. CMA and WES examinations due to structural fetal ultrasound abnormalities.

Organ system	Cases (n)	%
Fetal hydrops, cystic hygroma, thick nuchal fold	95	40.60
Craniospinal and craniofacial abnormalities	48	20.51
Cardiovascular abnormalities	42	17.95
Other thoracic abnormalities	6	2.56
Abdominal wall and abdominal abnormalities	7	2.99
Urogenital abnormalities	8	3.42
Limb anomalies and ossification disorders	14	5.98
Other structural ultrasound abnormalities	14	5.98
Total	234	100

Abbreviations: CMA = chromosomal microarray analysis; WES = whole exome sequencing.

In the remaining 105 cases (42%), abnormalities (deletions or duplications) were detected using CMA, 22% of which were confirmed as pathological abnormalities, and in 6 cases (2%) a trisomy affecting the entire chromosome 21 (in 5 fetuses) or chromosome 18 (in 1 fetus). The most common abnormalities and their locations are listed in Table 7. In three cases, the parents carried a balanced translocation, which caused an unbalanced status in the fetus: i) in the father, t(9;20)(p24;p12) and the fetus with a microdeletion in the 9p24.3p24.1 region and a microduplication of 14.755 Mb in the 20p13p12.1 region; ii) t(4;5)(p16;p15) in the mother and the fetus with 4.984 Mb microdeletion in the 4p16.3p16.2 region and 19.389 Mb microduplication in the 5p15.33p14.3 region; iii) t(4;8)(p16;p23) in the mother and a microduplication of 23.697 Mb in the 4p16.3p15.2 region and a microdeletion of 6.474 Mb in the 8p23-3p23.1 region in the fetus. In one case, we found a compound heterozygosity, which was identified by CMA as a 4.138 Mb (arr[GRCh38]12q12(38620776_42758565)x1) on the maternal allele and heterozygous deletion affecting 1 base in the KIF21A gene located in this region on the paternal allele using WES analysis. As a combined effect of the two abnormalities, severe pathological manifestations detectable already in the fetal stage were described in two affected fetuses in the family (asphyxiated thorax syndrome, nasal root edema, edema also observable on the fetal trunk, borderline cerebral ventriculomegaly).

Table 7. The most common copy number variations.

Copy number variation	Description	Cases (n)	%
16p11.2	Known LCR/SD region / VUS	23	30.26
3q24q29	Known LCR/SD region*	8	10.52
8p22p24.3	LOH polymorphic region**	8	10.52
16p13.11p12.3	Known LCR/SD region	7	9.21
Xq21.31q21	VUS	7	9.21
15q11.2q13.3	Known BP-(1–3) region*	6	7.89
4p16.3p16.2	Telomer region	5	6.58
9p24.3p24.3	Telomer region	4	5.27
22q11.21	DiGeorge region	4	5.27
20q13.33q14.33	Telomer region	4	5.27
Total		76	100

*LCR = low copy repeat; **LOH = loss of heterozygosity
BP = breakpoint; LCR = low copy repeat; SD = segmental duplication; VUS = variant of uncertain significance

In three cases, CMA was performed on DNA samples isolated from dead fetal tissue, and in one case, trisomy was confirmed on chromosome 18, and in another case, partial trisomy was confirmed on chromosome 9 (Table 5). In the testing sequence used by our working group, in cases where neither classical cytogenetics nor CMAs revealed any abnormalities, we attempted to map the known pathogenic point mutation using WES analysis as an additional test.

By the NGS performed we sequenced the entire human exome in 42 prenatal samples. Using WES testing, we identified pathological abnormalities in 9 cases (21.4%) that support heredity and are presumed to be related to the fetal phenotype according to the ClinVar database or the ACMG classification. Among the positive results, 2 cases were in the PTPN11 gene (c.922A>G p.Asn308Asp and c.923A>C p.Asn- 308Thr), one case in the SOS1 gene (c.1644T>A p.Ser548Arg), and one case in the EBP gene (c.338+1_338+2del). In another case, the affected mother carried the same arthrogryposis-associated variant as the affected fetus (TNNT3:c.188G>A p.Arg63His). In two cases, we observed a complex heterozygous status supporting autosomal recessive inheritance with severe ciliopathy (CC2D2A:c.4552C>T p.Arg1518Trp and c.4675-1G>C) and severe fetal akinesia associated with multiple arthrogryposis. In both cases, family segregation analysis confirmed the inheritance pattern. In two additional cases, the identified variants (CBL c.1754G>T p.Arg585Leu and TEK c.2744G>A p.Arg915His) were described in genes for which the phenotype associated with

the genes corresponds to the ultrasound findings, but the causal role of the variation is not clearly supported by the available data. By WES, we identified 18 cases of incidental/secondary findings that were classified as pathogenic or likely pathogenic according to the ClinVar database or the ACMG classification (Table 8).

Table 8. Presumed incidental findings in the prenatal WES examination group.

Gene	Variant	ClinVar classification	ACMG classification
ATAD3A	c.229C>G p.Leu77Val	P / VUS	LP
ANO5	c.2272C>T p.Arg758Cys	P / LP	LP
CFI	c.772G>A p.Ala258Thr	P / LP	LP
ROBO4	c.190C>T p.Arg64Cys	P	P
PROS1	c.701A>G p.Tyr234Cys	P / LP	LP
ANO5	c.692G>T p.Gly231Val	P / LP	LP
GATA4	c.487C>T p.Pro163Ser	P / VUS	LP
GATA4	c.34G>C p.Gly12Arg	VUS	LP
RPS7	c.298A>T p.Ile100Phe	–	LP
PAH	c.506G>A p.Arg169His	LP	P
F2	c.*97G>A	P / VUS	LP
ADGRV1	c.9623+1G>A	LP	P
F11	c.1693G>A p.Glu565Lys	LP / VUS	LP
BSCL2	c.974dupG p.Ile326fs	P / LP	P
PUS3	c.212A>G p.Tyr71Cys	P / VUS	LP
GJB2	c.35del p.Gly12ValfsTer2	P	P
CAPN3	c.550del p.Thr184ArgfsTer36	P	P
TPM3	c.253G>T p.Glu85Ter	–	LP

ACMG = American College of Medical Genetics and Genomics variant classification system (Franklin platform); ClinVar = ClinVar database classification based on current knowledge; LP = likely pathogenic; P = pathogenic; VUS = variant of uncertain significance; WES = whole exome sequencing.

In these cases, the phenotypic features associated with the genes did not clearly correspond to the ultrasound image, but all were associated with severe clinical manifestations.

4.3. Presentation of the clinical usefulness of detailed neurosonography through two cases

4.3.1. Case 1

4.3.1.1. Patient information and obstetric history

A 46-year-old expectant mother attended the first trimester screening when 12 weeks and 5 days pregnant. Conception was achieved by IVF with oocyte donation (the donor was under 25 years of age). She had miscarried during a prior pregnancy at gestational week seven.

Her medical history included antiphospholipid syndrome, hypothyroidism, and insulin resistance, and she regularly took metformin, levothyroxine, liothyronine, progesterone, escitalopram combined with clonazepam, and enoxaparin. There were no genetic diseases in her family. Her body mass index (BMI) was within the normal range.

4.3.1.2. Ultrasound results

The extended first trimester ultrasound examination (NT, nasal bone, DV, tricuspid valve regurgitation, early cardiac and intracranial examination) showed no abnormalities. The nuchal translucency was 2.4 mm, which corresponded to the 85th percentile based on the measured CRL of 69.9 mm, which was 3 days more than the gestational age calculated according to the IVF.

Mild polyhydramnios was diagnosed in the second trimester (deepest vertical pocket: 9.3 cm, amniotic fluid index: 21 cm). BPD (52.2 mm) and HC (191.1 mm) were above the 90th percentile, with all other biometric data within the normal range. The diameter of the posterior horn in the lateral ventricle atrium was 9 mm. The ambient and quadrigeminal cisterns were observed to be slightly dilated (Figure 1), while the Sylvian fissure was measured at 10.5 mm, found to be above the 95th percentile (Figure 2a) in respect of the normal range published by Alonso et al. [55] In addition, mild hypertelorism (external-external distance: 35 mm, internal-internal distance: 16 mm) and echogenic intracardiac focus (EIF) in the left ventricle, as well as mild bilateral pyelectasis were identified. The pediatric cardiologist conducted an echocardiogram, which showed no cardiac abnormalities.

Fetal follow-up scans were conducted at 27, 31, and 34 weeks of gestation. Mild to moderate polyhydramnios was confirmed during pregnancy. Meanwhile, BPD and HC recorded a steady increase, and by the 27 weeks of gestation, both exceeded the 99th percentile.

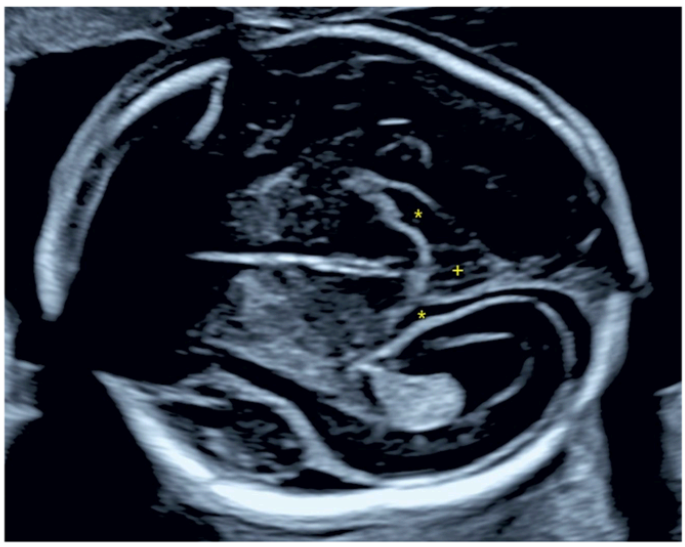
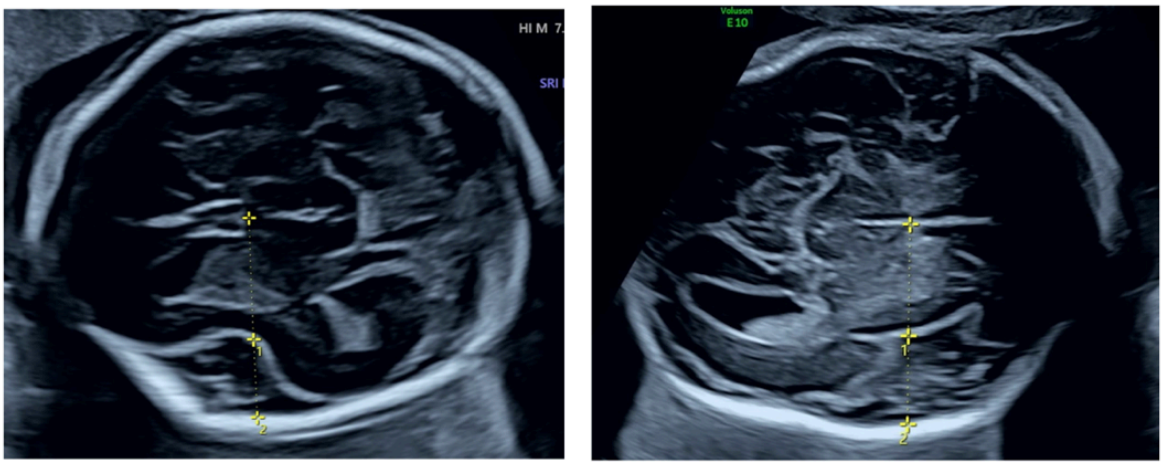


Figure 1. The enlarged ambient cisterns (*) and quadrigeminal cistern (+) at the 20th gestational week.

The ambient and quadruple cisterns stayed enlarged, while the subarachnoid spaces were widened. At 27 weeks of gestation, the Sylvian fissure was 17.4 mm deep, significantly exceeding the 95th percentile (Figure 2b), and external hydrocephalus was visible on the top view of the skull (Figure 3). At 27 weeks, the AC was in the 98th percentile and the estimated fetal weight was in the 97.9th percentile. These biometric data remained unchanged during pregnancy. The right renal dilatation remained mild, with a diameter of 8 mm at 34 weeks of gestation, while the right renal pelvis width normalized.



(**a**) (**b**)

Figure 2. Measurements of the Sylvian fissure. Measurements noted with 1 show the insular depth, measurements noted with 2 show the Sylvian fissure depth. (**a**) The depth of the Sylvian fissure was measured at 10.0 mm at the 20th week; (**b**) and 17.4 mm at the 27th week. Both measurements were above the 95th percentile in respect of the database published by Alonso et al. in 2010 [16].

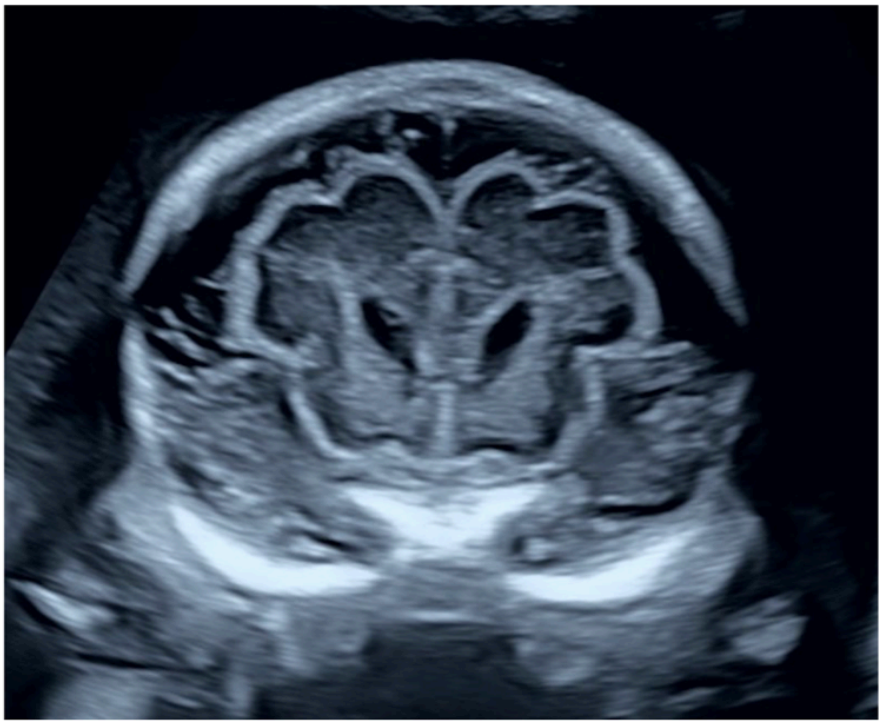


Figure 3. Coronal transcaudate plane in the 27th gestational week. Clear dilation of the subarachnoid spaces.

4.3.1.3. Genetic testing

A cf-DNA test was conducted in the first trimester, yielding normal results. Following the second trimester examination, amniocentesis was performed. G-band analysis of the cell culture obtained from amniocentesis did not reveal any chromosomal abnormalities (46XY).

Using array CGH, a 2 Mb heterozygous microdeletion affecting the RAB6C (OMIM: 612909) and SMPD4 (OMIM: 610457) genes was confirmed. Subsequent whole-exome sequencing ruled out complex heterozygosity but identified a heterozygous pathogenic variation in the SOS1 gene (NM_005633.4:c.1644T>A, p.Ser548Arg), confirming Noonan syndrome 4 (phenotype MIM number: 610733, location: 2p22.1, gene/locus MIM number: 182530).

4.3.1.4. Perinatal and postnatal outcome

Following extensive genetic counseling, the expectant mother opted to continue with the pregnancy. Her baby was delivered by cesarean section at 38 weeks of gestation, and a birth weight of 3530 g and an Apgar score of 9/10 were recorded.

Routine neonatal vision and hearing tests yielded normal results. The baby received regular cardiological check-ups, revealing mild supravalvular pulmonary stenosis, which did not progress during the control period. The last check-up was at the age of 2 years, no intervention was necessary, and annual check-ups are planned.

A cranial ultrasound performed at 7 weeks postnatally showed no abnormalities. The neurological examination showed minimal hypotonia along with moderately delayed psychomotor development. Accordingly, complex (motor, mental, speech) rehabilitation was suggested, and a 12-month follow-up examination was scheduled.

4.3.2. Case 2.

4.3.2.1. Patient information and obstetric history

A 32-year-old expectant mother presented to our clinic with her second pregnancy, conceived spontaneously. Previously, following an uneventful pregnancy, she had undergone a normal vaginal delivery at 39 weeks of gestation. Aside from hypothyroidism, which did not require treatment, she had no chronic conditions.

There were no known hereditary diseases in her family, and her BMI was within the normal range.

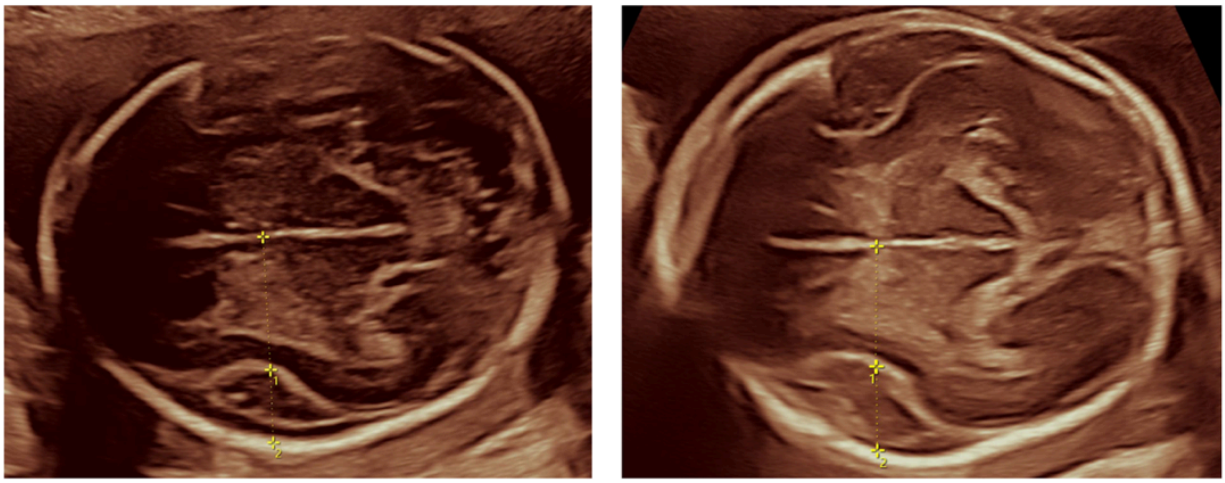
4.3.2.2. Ultrasound examination results

The first trimester screening was conducted at a different clinic, where the NT value was 3.2 mm and DV agenesis was suspected; at this point the patient was referred to our clinic. Here the NT value was recorded as 3.4 mm, which corresponds to the 99th percentile based on the CRL of 70.6 mm. DV agenesis, SUA, EIF in the right ventricle, and frontal depression in the skull bones were also reported.

A follow-up ultrasound examination conducted at 16 weeks of gestation provided confirmation of these anomalies as well as DV agenesis (intrahepatic portosystemic shunt).

Fetal echocardiography conducted by a pediatric cardiologist did not reveal any significant cardiac abnormalities, but a small subaortic ventricular septal defect (VSD) and suspected septal membrane aneurysm were identified. At 21 weeks, abnormal PFSR and ventricular hypertrophy were also identified.

At 19 weeks, an enlarged Sylvian fissure measuring 8.0 mm was identified, but otherwise the neurosonography was normal. At 22 weeks of gestation, the Sylvian fissure was 10.9 mm deep (Figure 4). Both measurements were in excess of the 95th percentile. Enlargement of both the subarachnoid spaces and the Sylvian fissures was also obvious in the coronal plane (Figure 5).



(a) (b)

Figure 4. Measurements of the Sylvian fissure. Measurements noted with 1 show the insular depth, measurements noted with 2 show the Sylvian fissure depth. (**a**) The depth of the Sylvian fissure was recorded as 8.0 mm at the 19th week; (**b**) and 10.9 mm at the 22nd week. Both are above the 95th percentile based on the database published by Alonso et al. in 2010.

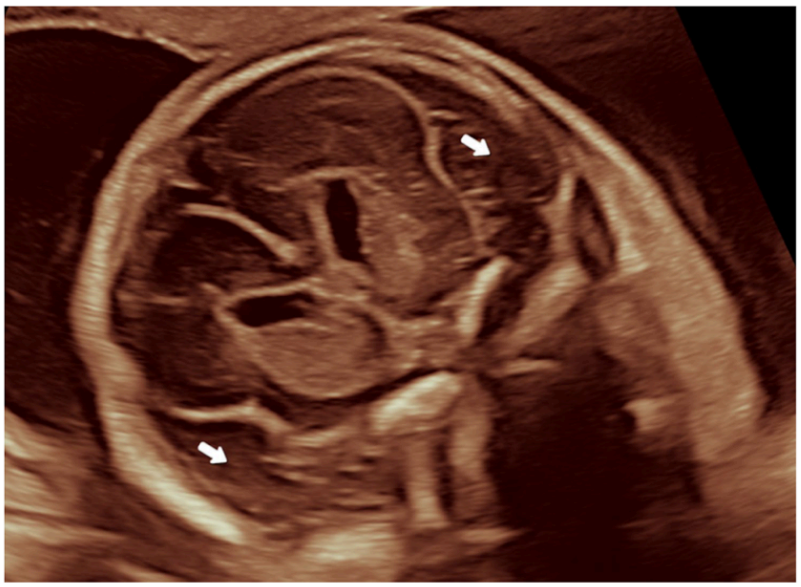


Figure 5. Coronal transcaudate plane in the 22nd gestational week. Significant dilation of the subarachnoid spaces and Sylvian fissures visible (arrows).

4.3.2.3. Genetic testing

No cfDNA testing was done. An amniocentesis was conducted; karyotype analysis and SNP microarray results showed no abnormalities. Whole exome sequencing revealed a heterozygous variation in the *PTPN11* gene (c.124A>G, p.Thr42Ala; chr12-112884189 A>G, NM_002834.5, rs397507501), thus confirming Noonan syndrome 1 (phenotype MIM number: 163950, location 12q24.13, gene/locus MIM number 1768762.2.4).

4.3.2.4. Perinatal and Postnatal Outcome

After extensive genetic counseling and full consultation, the expectant mother opted to terminate the pregnancy.

5. Discussion

5.1. Experiences in first trimester echocardiography and the learning curve for the early heart scan

According to clinical data for unselected expectant mothers screened at our clinic the study population can be described as a medium risk profile in terms of maternal age. The average age between the entire population and the CHD group was not significant, but the proportion of people over 35 was significantly higher in the CHD group.

These results support the suggestion that rises in the frequency of congenital anomalies, including CHD, may be related to advancing maternal age and the inferred risk of chromosomal abnormalities. [56,57] This study showed that fetal karyotype abnormalities occurred in 24% of studied cases (9/37). It should indeed be observed that advanced maternal age contributes significantly to fetal heart development abnormalities (mainly via increased aneuploidy rate). Complications for mothers and fetuses increase significantly when the mother is over 35 years of age during pregnancy. Recent studies have linked Maternal age over 35 to subclinical myocardial dysfunction [58] and a number of obstetric complications (gestational diabetes, gestational hypertension, preeclampsia). [59–61]

At the same time, it can be observed in Hungary that expectant mothers over 35 years of age from higher socioeconomic backgrounds are more likely to take advantage of market-leading screening and diagnostic obstetric services at expertly-staffed, specialized obstetric clinics in the private sector; accordingly, detection rates across a range of fetal pathologies are significantly higher than the overall mean for this age group. Finally, it is important to note that primary care obstetricians routinely and promptly refer high-risk patients to these perinatal clinics.

Recent studies have indicated that among routinely screened fetuses, those with NT values in excess of the 99th percentile (threshold > 3.5 mm) should be considered as a new high-risk group. Several studies have demonstrated that additional markers of first-trimester screening for aneuploidy, including TR and DV, might also be relevant in congenital heart defect screening. In combination, NT measurement and TR and DV blood flow assessment in the first trimester results in a 48% detection rate of severe congenital heart defects. [62,63]

Measurements of NT, DV, and TR in this study reinforce observations that the introduction of these new cardiac markers into extended first trimester screening can enable a more precise definition of CHD-prone fetuses, beyond those with classic indications and high baseline risk. However, that is not to suggest the introduction of routine clinical use of NT, DV, and TR will result in detection all congenital heart defects. [30,61,63,64] For detection of most CHDs, it is also crucial to perform structural examination of the fetal heart in the three at-risk groups: maternal age over 35; abnormal NT, TR, and DV-related risk pregnancies; and CHD with high baseline risk and classic indications.

Screenings were performed with the newest, most advanced ultrasound equipment. Rather than high-frequency linear probes specifically designed for cardiac examination, mid-frequency, high-resolution convex abdominal transducers were used, as their wide viewing angle and increased tissue penetration was more suited to the needs of a full first-trimester ultrasound screening. In cases of retroflexed uterus, transvaginal ultrasound probes were used, but outside of these cases this method was rarely used, on account of its restricted range of movement. Experience at our center reinforces the finding that visualization quality is not directly dependent on body mass index (BMI), but rather on the distance between the transducer and the fetal heart, which may be affected by several factors including BMI, the position of the uterus, placenta, and fetal position. [29–31,65,66]

Extended first trimester screenings were conducted by three obstetricians, each of whom had intermediate ultrasound experience of between 4 and 8 years in fetal diagnostics (predominantly in the second trimester) and had completed a 12-month FMF-TR/DV audit before the start of the study (2010). The three obstetricians had only limited experience in evaluating healthy versus abnormal fetal hearts; however, At the beginning of the study period, their limited experience was compensated for in terms of the greater time given to the examination, together with offline analysis of up to 3–4 hours in some cases. Later, the addition of a highly trained ultrasound technician to the team enabled obstetricians to concentrate on evaluating abnormal findings, reducing the total duration of the examination to 20–30 minutes. After screening by the ultrasound technician, an obstetrician was always required to perform a follow-up screening, preferably using checklists, to guarantee quality control throughout the study.

Prior to this study, the pediatric cardiologist at our center had only performed echocardiography on fetuses older than 16 weeks; however, with a positive attitude to

professional development, this clinician gained experience and confidence performing ultrasound examinations in the first trimester.

Following the study period, those fetal hearts classified as “abnormal” by obstetricians with moderate levels of experience were re-examined by the pediatric cardiologist, with pregnant mothers receiving a full fetal cardiology second opinion inside 24 hours, with the aim of reducing anxiety.

Case 36 highlights the new finding that CHD progression can be rapid, in particular during the first 20 weeks of pregnancy.

During the initial learning curve period (2010–2012), the nine CHD cases detected in the first trimester of pregnancy were typically and frequently characterized by an abnormal four-chamber view and the inability to detect implication of the major vessels in the heart defect. (All studies dealing with prenatal ultrasound examination of abnormal hearts, including the present study, are affected by the limitation that it is not possible in most cases to perform a subsequent histological examination of the fetal heart during the first trimester of pregnancy). Notwithstanding that the examination of the outflow tracts did not form part of the CHD diagnosis, it was found to be useful. The extended time frame offered for extended screening in the first trimester greatly improved the efficiency of transducer manipulation, and contributed to empirical learning.

As shown in Table 1, during the second part of the study (2013–2014), abnormal large vessel diagnoses were also observed in the first trimester (tetralogy of Fallot, TGA).

5.2. The first Hungarian experiences concerning prenatal chromosomal microarray analysis and whole-exome sequencing

During CMA testing, we confirmed microdeletions or microduplications in fetal samples in 105 cases. The large number of cases also confirmed that the genomic structural forms described in the literature can influence the frequency of microdeletions and microduplications. Such structural elements include, for example, so-called "low copy repeat" (LCR)/segmented duplication regions: these are repetitive sequences of 10–300 Kb in size, which can form microdeletions or microduplications in the daughter cell during meiotic division in the process of non-allelic homologous recombination (NAHR). [67]

For example, according to the literature, LCRs are found in the p13.1, p12.3, p12.2, p11, q22.2, and q23 regions of chromosome 16, which can cause rearrangements of varying sizes and different breakpoints during meiotic division. Although these LCRs are located in the so-called gene-poor environment of the pericentromeric region of chromosome 16, depending on the genes involved, they can still lead to abnormal phenotypes in certain cases. In the fetal samples we examined, we detected the 16p11.2 microdeletion or microduplication located in this region most frequently (23%). Based on the literature, this variation is of uncertain or variable clinical significance (VUS category). [68]

Segmental duplications (the best-known form of these is the tandem duplication) account for more than 6% of the genome. These repetitions can also cause structural changes in the genome, both through NAHR and non-homologous end joining (NHEJ). As a result of the latter, the duplicated DNA fragment—moving to an extrachromosomal position and then reinserting into the genome—can create DNA excess or deficiency, or, if it affects a single gene during insertion, a monogenic pathogenic phenotype. [69] In our fetal samples, we found the fourth most common (4%) occurrence of excess or deficiency in the well-known 22q11.21 region, which is also associated with the presence of the above-mentioned LCR segmented duplication regions in the background of microdeletions or microduplications. Among the samples examined, we confirmed both deletions and duplications: the confirmed microduplication affected the LCR22-2 (A) and LCR22-3b (C) regions.

In postnatal cases, the clinical phenotype was characterized by developmental and intellectual retardation, physical disorders such as hypotonia, moderately dysmorphic facial features, and microcephaly. The confirmed fetal deletion cases corresponded to 22q11.21 deletion syndrome (DiGeorge/VCF). In these cases, the proximal and central regions were affected (LCR22-2 [A] – LCR22-5 [E]). [70,71] Any CNV (microdeletion or microduplication) in the so-called critical region of chromosome 15, 15q11.2-q13.1, the so-called critical region, any CNV (microdeletion and microduplication) of BP1–BP3 and BP2–BP3 may pose a risk for developmental disorders and autism spectrum disorders. [72,73]

We found alterations in this region in 6% of the fetal samples examined, which may be associated with neuropsychiatric disorders. In cases where balanced translocation was detected in the parents using classical cytogenetic methods (G-banding), invasive sampling is necessary in all cases, in addition to fetal ultrasound abnormalities, as parental balanced translocation can easily become unbalanced in the fetus. In the population studied, the parents carried a balanced

translocation in three cases, and in all three cases this caused an unbalanced state in the fetal samples, which was associated with a pathological phenotype.

Cases in which so-called "complex heterozygosity" is the cause of the pathological phenotype should be highlighted. In one case, we confirmed compound heterozygosity with CMA and WES tests, which was also accompanied by a severe pathological fetal ultrasound image: in the affected fetal sample, we found a heterozygote 4.138 Mb deletion inherited from the mother and a single-base deletion located in this region inherited from the father, which, as a compound heterozygote, was associated with a severe phenotype already in the fetal period. Random events may be behind complex heterozygosity, which is why it is important to rule out the potential presence of point mutations by sequencing in cases where CMA testing has confirmed a heterozygote deficiency or excess and an autosomal recessive gene is also involved.

In cases where neither classical cytogenetics nor CMA testing confirmed any pathological abnormalities, we performed WES testing as a next step. [40,74] During our tests, we identified a presumed pathogenic variant in a gene corresponding to the fetal phenotype in 9 cases, and a variant involving amino acid substitution in a gene associated with Noonan syndrome in 3 cases and Noonan-like syndrome in 1 case (PTPN11, SOS1, CBL).

At the family segregation studies, we found that the latter variant was also carried by the parents, so we modified its classification. In connection with skeletal abnormalities, we found a presumed pathogenic variant in three genes (TNNT3, KIF21A, ABP). In one case, the autosomal dominant phenotype was confirmed by the fact that the symptomatic mother also carried the variant, while in the other case of a dominant phenotype, the variant was likely to be of de novo origin. In the third case, the child inherited the identified variants in a complex heterozygous form from the carrier parents. In the case of the fetus with cardiological symptoms, the pathogenic role of the identified variant was not confirmed. Based on the literature, a causal relationship can be assumed, as the variant we detected in the TEK gene had previously been identified in a family affected by VMCM (venous malformations, multiple skin and mucosal abnormalities), where the mother was also affected. In our case, the family refused further testing.

In one case, based on fetal ultrasound examination, we identified two presumably pathogenic variants in the CC2D2A gene in a complex heterozygous form with suspected

ciliopathy, where the trans-location of the variants was confirmed by family segregation analysis. When communicating the results, the evaluation is primarily based on the existing phenotypic features, even in the case of fetal testing. Since this information is only indirectly or limitedly available to us prenatally and may change during pregnancy, it is advisable to further narrow down the list of variants. This can be done by looking at the inheritance pattern of diseases associated with the affected genes, the typical time of onset of the diseases, or the severity of the phenotype. [52,75]

Variants of uncertain significance (VUS), incidental and secondary findings also pose a serious challenge for laboratories and clinicians. Incidental findings are variants identified independently of clinical indications. Secondary findings are variations included in the gene list compiled and validated by ACMG experts that are not related to the primary phenotype but may provide information that can be used in the medical decision-making process. Among those we examined, we identified 18 cases of pathogenic or likely pathogenic variants in the ClinVar database or according to the ACMG classification in association with an autosomal dominant disease pattern. [76] For the identified variants, we examined the phenotypic characteristics, the literature data, and, where possible, the parental samples. [77]

During the review, we were able to classify the identified variants into larger groups. Most of the data on the pathogenicity of the variants we identified were reported in the context of autosomal recessive disease, and in some cases, the autosomal dominant role was not yet sufficiently supported (e.g., BSCL2, PUS3, GJB2, CAPN3, TPM3, ADGRV1, ANO5, and ATAD3A genes). For these genes, clinical re-evaluation, repeated analysis of the fetal phenotype, comparison of different molecular techniques, family segregation studies, and a review of the literature may help in deciding whether to report the results. [78–80]

5.3. Presentation of the importance of detailed neurosonography through two cases

NS is a frequently occurring genetic disorder with a wide range of genotypes and phenotypes. This diversity presents a challenge not only to prenatal but also postnatal diagnosis.

While it is possible to detect NS using a variety of genetic testing methods including targeted Noonan panels, WES, and cf-DNA tests, not all national health systems routinely offer screening tests and may not be financially viable for all patients. Partly for these reasons, diagnostic tests are often only performed when it is clearly indicated. Consequently,

sonographic markers contribute effectively to prenatal screening by providing early indicators of suspicion leading to improved detection rates. [81] Suspicion of Noonan syndrome arises primarily following detection of increased NT in the first trimester. [82,83] An important caveat is that the personal preferences and socioeconomic profile of the patient may affect access to first-trimester screening. [84–86]

In a 2024 review publication, Tangshewinsirikul et al. observed that elevated NT was the most commonly detected prenatal ultrasound anomaly among 105 cases of Noonan syndrome, accounting for 71% of cases. Second was various body fluid accumulations (59%), and polyhydramnios was third (50%). [87] However, a study involving 151 children with NS found only 12.5% of echocardiograms to be normal, with 62% reporting pulmonary stenosis. Among 105 fetuses reporting NS, results showed ventricular hypertrophy to be the most commonly reported, occurring in 33% of cases, while pulmonary stenosis occurred in only 13%. [87,88]

Intracranial cases of NS are reported predominantly in children or young adults and include hydrocephalus, Chiari I malformation, diverticular enlargement of the foramen of Luschka, and cerebrovascular aneurysms. [80–82] Helenius et al. described a fetus at 22 weeks in whom prenatal MRI revealed NS with RAF1 variation, enlarged extracerebral CSF, delayed operculization, and hypoplastic vermis. [89] Gripp described widening of the subarachnoid spaces in children with SHOC2, while Zarate described it in individuals with NS with RAF1 variants, as well as other extra- and intracranial abnormalities. Mastromoro et al. made an MRI diagnosis of EH at 27 weeks of gestation following a genetic diagnosis of NS resulting from a PTPN11 gene variation. Here, the size of the corpus callosum and cerebellar vermis was below the 10th percentile. [90] EH has been previously diagnosed by MRI prenatally in disorders including Snijders Blok–Campeau syndrome and benign external hydrocephalus. [91,92] Baron et al. detailed 21 cases of macrocephaly diagnosed by ultrasound, prompting fetal MRI, by which the presence of EH was confirmed in 77% of cases. [93]

Postnatally, enlargement of the subarachnoid fluid spaces has been associated in the literature with RASopathies. [94] In addition to the SOS1 gene variation, a postnatal central nervous system anomaly described was corpus callosum agenesis with severe developmental delay. [95] Another case study detailed spinal cord lesions similar to plexiform neurofibromas in NS resulting from variation in the SOS1 gene. [96]

The present study identified two gene variants, SOS1 and PTPN11, as playing a role in the development of the central nervous system. This role has been described in previous studies. The PTPN11 gene encodes the Shp2 protein and shows a gain-of-function variation in NS. In mice, Shp2 causes a reduction in axonal myelination and an increase in neuron density, while reducing astrocyte density in the forebrain and hippocampus. Meanwhile, SOS1 stimulates nerve growth factor, which in newborns is expressed in the cerebral cortex, while activating the Ras-MAPK pathway NMDA receptors, also in the cerebral cortex. [97,98] Further investigation is needed to establish if, and via which pathway, these genes contribute to the development of EH in NS.

In the two cases reported on by the present study, EH presented in the second trimester without any other significant central nervous system (CNS) abnormalities and was confirmed by ultrasound examination and measurement of the depth of the Sylvian fissure in the axial plane. To our knowledge, based on the available literature, case 1 is the first to describe external hydrocephalus in which the central nervous system is otherwise normal, with a variation in the *SOS1* gene. Moreover, these comprise the first cases of NS in which EH was diagnosed by ultrasound in the second trimester.

Based on ISUOG guidelines, fetal MRI is prompted in 7–15% of cases after a well-conducted neurosonogram. For the present study, ultrasound alone was found to be sufficient for evaluating the subarachnoid spaces, as it enabled greater cost-effectiveness, quicker diagnosis, and reduced demand on human resources relative to MRI. Notwithstanding the two cases highlighted, it should be noted that EH could have developed independently of NS in both cases. Nonetheless, as external hydrocephalus is a recognized occurrence in children with NS, it can be fairly assumed that the prenatal detection is connected to the syndrome. [89,99,100]

Examination of the subarachnoid spaces does not at the present time form part of routine screening, which may be a factor in the number of undetected cases of external hydrocephalus. Based on this study, the authors recommend measurement of the depth of the Sylvian fissure for the second trimester prenatal diagnosis of external hydrocephalus. Moreover, as a complementary marker, it may help to ensure prompt performance of genetic testing, a point of relevance concerning legal regulations in some countries on termination of pregnancy. While measuring the depth of the Sylvian fissure in this respect requires more

investigation, it can be readily incorporated into routine screening, as its technical implementation is not dependent on additional training or skills.

5.4. Integrated re-interpretation of the results within the cardio–neuro–genetic framework

The results of the three original studies presented in this thesis can be interpreted within the integrated cardio–neuro–genetic framework introduced in previous chapters. This multidimensional approach reflects the interconnected nature of fetal cardiovascular, central nervous system, and genetic development, emphasizing that abnormalities in one part of these frequently co-exist with abnormalities in the others. The implementation of this framework highlights a paradigm shift from isolated organ-focused diagnostics toward a systemic, complex evaluation strategy capable of identifying fetal pathologies early enough to comply with the legislative requirements in Hungary.

Within this integrative model, our first-trimester echocardiography study demonstrates that early structural cardiac evaluation by moderately experienced obstetricians can approach the diagnostic accuracy of pediatric cardiologists, provided that proper training, protocol adherence, specialist feedback and quality control are maintained. This finding supports the concept that early cardiovascular screening not only detects structural heart anomalies but also serves as an entry point to the broader cardio–neuro–genetic continuum. In practice, an abnormal early cardiac finding may indicate a higher probability of concurrent chromosomal abnormalities or central nervous system anomalies. Similar observations have been made in the literature, where cardiovascular malformations frequently correlate with syndromic or neurodevelopmental outcomes [16,27,31].

The second major component of this thesis, our Hungarian experience with chromosomal microarray and whole-exome sequencing, contributes the genetic dimension of this framework. The high diagnostic yield achieved after negative karyotyping underlines the important role of subchromosomal and nucleotide-level analyses in clarifying the etiology of fetal abnormalities detected by ultrasound. When combined with detailed phenotypic information from cardiac and neurosonographic assessment, these tools refine the accuracy of prenatal counseling and prognosis. A recent meta-analyses confirmed that over 20% of fetuses with complex cardiac or CNS malformations show clinically relevant variants detectable by WES [52,53].

The third cornerstone of this integrative evaluation was illustrated by the two Noonan syndrome cases associated with prenatally confirmed external hydrocephalus [107]. These cases exemplify the neurodevelopmental manifestation of a genetic disorder. The identification of SOS1 and PTPN11 variants in fetuses with both subtle cardiac markers and CNS abnormalities supports the hypothesis that certain gene mutations express phenotypic effects through interconnected pathways (involving the Ras-MAPK signaling in these cases). The recognition of external hydrocephalus as a potential prenatal sign of RASopathies expands the neurosonographic phenotype of these.

The clinical importance of the complexity built by the three parts of the cardio-neuro-genetic framework becomes particularly clear when considering their temporal and diagnostic interactions. Early echocardiography defines the cardiovascular phenotype, while neurosonography adds the assessment of brain maturation and structural development. When these imaging modalities are supplemented by tiered molecular diagnostics (karyotyping, CMA, WES), the combined approach shortens the time to diagnosis and enhances the interpretive context of each finding. The fetal phenotype thereby guides the depth of genetic evaluation. Recent international consensus guidelines also recommend this sequence - ultrasound led phenotype characterization followed by genetic testing - to optimize diagnostic efficiency and parental counseling [94,97].

From a methodological perspective, the integration of these three parts also redefines the clinician's role in fetal medicine. The fetal medicine specialist is no longer an isolated obstetrician-geneticist but a coordinator of a multidisciplinary diagnostic process. Since it is not always possible for one person to perform a detailed examination of all target organs, and it is not even necessarily expected, it is important to emphasize the importance of teamwork. As shown by the learning curve analysis, appropriate training and iterative feedback loops between obstetricians, fetal cardiologists can yield an accuracy approaching 90% for major congenital heart disease in an unselected population. This model can be generalized to neurosonography and genetic diagnostics, where shared image databases, standardized protocols, and relevant decision-support tools are emerging. Future incorporation of artificial intelligence into image interpretation and genotype–phenotype correlation is expected to strengthen this integrative model further [48,98].

In the Hungarian context, where legal time limits for pregnancy termination necessitate timely and precise decision-making, the cardio–neuro–genetic framework offers both medical

and ethical advantages. By linking cardiac, neural, and genetic findings, clinicians can provide comprehensive counseling within the legally available window, facilitating informed parental decisions. Furthermore, early detection of multi-systemic abnormalities allows better perinatal planning, including targeted neonatal intensive care and surgical readiness for complex congenital defects. These aspects align with the principles of predictive and personalized prenatal medicine as defined in recent papers [49].

Finally, the cardio-neuro-genetic framework underlines the principle: fetal development is a networked process, not a collection of independent organ systems. The heart, brain, and genome interact through overlapping molecular and hemodynamic pathways. Disturbance in one component may cascade across others, producing the complex phenotypes observed in congenital disorders. The integration of cardiovascular imaging, neurosonography, and genetic technologies represents not only a diagnostic synergy but a conceptual advance toward understanding the fetus as an integrated biological system. Within this view, the cardio–neuro–genetic model can serve as a foundation for future interdisciplinary education, data integration, and translational research in fetal medicine.

6. Conclusions

Based on the results of this study, detection rates in first-trimester fetal cardiovascular ultrasound screening performed by moderately experienced obstetricians may be improved to 90% in the unselected “routine” pregnant population for severe congenital heart defects, provided the appropriate apparatus, quality control measures, and motivation are in place. The data show that early fetal echocardiography in most cases provides reassurance to pregnant women simply by ruling out the most common congenital abnormalities and heart defects. At the same time, it also helps to prompt early diagnosis of chromosomal abnormalities. Most fetal heart defects detected are typically of a high level of complexity and severity. Accordingly, couples may choose to terminate the pregnancy early, also reducing overall maternal health risks as well as the psychological stress of elective termination in the second trimester. Cardiac markers used in the first trimester (NT, TR, and DV) can help to indicate severe, complex structural heart defects and can also inform the detection of both minor and major fetal heart defects that are only visible during second-trimester screening. This represents the potential for improvements in neonatal morbidity and mortality by informing the timely setup of intensive perinatal cardiac care.

Confirmation via fetal echocardiography conducted by pediatric cardiologists to reevaluate suspected cardiac abnormalities reported by less experienced obstetricians is also important for helping genetic counselors and couples gain an improved understanding of the disease in terms of its severity, complexity, and prognosis. In this way, the psychological stress for parents is greatly reduced. This study involved a five-year learning curve, within which nearly 4,800 first-trimester fetal cardiac examinations were performed, over 40 fetal heart abnormalities were identified, and physicians’ proficiency in visualizing and evaluating the four-chamber view and outflow tract planes, together with the major vessels of the fetal heart at 11–13 weeks was significantly improved. It is reasonable to conservatively assume that an experienced obstetric sonographer conducts 1,300 first-trimester aneuploidy and cardiovascular screenings per year. This means that just 10–15% of moderately and highly experienced obstetric ultrasonographers in Hungary would need special training to be able to conduct detailed early fetal echocardiography and thus guarantee access to all pregnant women in Hungary.

With a structured, high-quality training program, this goal could be achieved in less time than the learning curve of this study, meaning that first-trimester fetal echocardiography could be incorporated into routine first-trimester screening within five years or less.

In prenatal testing, high-resolution methods such as CMA and WES testing are becoming increasingly popular alongside classic cytogenetic testing. Based on international protocols and our own experience, CMA testing has proven to be useful and quick to perform in cases of positive ultrasound and/or NIPT results with a stressful medical history. The CMA method is also suitable for accurately determining the origin of extra chromosomes that have been confirmed by classical cytogenetic testing but cannot be determined or are difficult to determine using banding techniques. It can also be reliably used to determine the origin of extra marker chromosomes and to identify any unbalanced abnormalities that may develop in the fetus in cases of balanced translocations detected in the parents.

In cases of miscarriage, the CMA method can be used in cases where cells cannot be examined using classical cytogenetic methods, as the cells may already be in a necrotic state and unable to divide. Based on our experience, DNA isolation from the abortion must be performed shortly after fetal death has been confirmed by ultrasound, as the onset of possible necrotic processes may affect the effectiveness of the CMA test. Taking into account international recommendations, we reported these findings as follows: i) we reported all pathological and highly probable pathological abnormalities in all cases, ii) based on size, we reported microduplications larger than 1 Mb and microdeletions larger than 0.5 Mb, iii) according to the ACMG standard, the pathogenicity of a given abnormality can also be determined based on the point value calculated for CNV, iv) we also used databases (Decipher, ClinVar, ClinGen) to provide phenotype predictions based on postnatal cases described in the affected region and characteristics specific to the affected genes (e.g., whether the affected gene is dose-sensitive or not). The indications for WES testing are not yet as broad as those for CMA testing, but WES is recommended in all cases where a genetic origin is likely and preliminary conventional karyotyping and CMA testing have yielded negative results. Where the advanced stage of pregnancy makes it necessary, it is advisable to initiate CMA and WES testing simultaneously. In cases where family history or fetal phenotype suggest a nucleotide-level genetic background, WES may precede CMA [6, 30].

Overall, CMA provided additional information in 22% of cases (252 cases), while WES provided additional information in 21.9% of cases (42 cases) compared to classical

cytogenetics. Publications in the international literature give different percentages for this: 6–10% and 20% for CMA [31, 32], while for WES the lowest is 6.2% and the highest is 57.1% [33]. The differences may be due to the use of different platforms: in the case of CMA, the higher-resolution array is able to detect more abnormalities, and different percentages can also be obtained by selecting the size of the abnormalities reported in the findings. G-banding, CMA, and WES can identify genetic involvement in approximately 35% of cases, while unidentified genetic, epigenetic, multifactorial, environmental, or teratogenic effects may be behind the remaining 65% of abnormal ultrasound findings and fetal developmental abnormalities. Based on this, our working group considered it important to develop a uniform prenatal diagnostic protocol for CMA and WES testing, taking into account international recommendations and adding national characteristics. It is important to note that neither CMA nor whole genome sequencing methods are capable of detecting or completely ruling out all genetic disorders. The residual risk of rare disorders, especially in cases of multiple congenital anomalies, is an important consideration during counseling.

The results of this thesis demonstrate that the cardio–neuro–genetic framework provides a coherent and clinically applicable model for understanding the complexity of fetal development and disease. The integration of early echocardiographic, neurosonographic, and genetic data establishes a pathway from phenotype to genotype that enhances diagnostic precision, accelerates clinical decision-making, and supports more individualized patient counseling.

First trimester extended echocardiography, when performed under standardized conditions by trained obstetricians, can achieve excellent diagnostic accuracies. When linked to genetic testing such as CMA and WES, these imaging results gain deeper explanatory power by revealing the underlying molecular mechanisms of observed structural changes. Similarly, detailed neurosonography expands the prenatal phenotype, allowing detection of subtle cerebral anomalies, as shown in the presented Noonan syndrome cases. Together, these findings show that cardiac, neurological, and genetic evaluations are not isolated diagnostic tools but complementary dimensions of a complex developmental network.

This integrated model has practical implications for the organization of prenatal care. It supports a multidisciplinary workflow where obstetricians, pediatric cardiologists, neurosonographers, and clinical geneticists collaborate in a structured diagnostic pathway. The resulting synergy improves detection rates, reduces diagnostic delays, and allows the

implementation of targeted perinatal management strategies. On a broader scale, it represents a step toward individualized fetal medicine, aligning prenatal diagnostics with the personalized approaches that already define postnatal healthcare.

Beyond its clinical relevance, the cardio–neuro–genetic framework also carries educational and ethical value. It promotes a unified understanding of fetal pathology that encourages the cross-training of specialists and the standardization of protocols, while ensuring that parents receive transparent, evidence-based counseling. By fostering integration rather than segmentation, this approach strengthens both the scientific and human dimensions of prenatal diagnostics.

In summary, the findings of this thesis suggest that the cardio–neuro–genetic paradigm not only enhances the detection and interpretation of complex congenital anomalies but also provides a conceptual foundation for individualized care in fetal medicine. Its application may lead to more efficient national screening systems, improved interdisciplinary collaboration, and ultimately, better perinatal outcomes for the families.

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Appendices

Appendix No. 1.

Co-author certification

I, myself as a corresponding author of the following publication declare that the authors have no conflict of interest, and Tibor Elekes Ph.D. candidate had significant contribution to the jointly published research. The results discussed in her thesis were not used and not intended to be used in any other qualification process for obtaining a PhD degree.

The publication relevant to the applicant's thesis:

Summary of the first Hungarian experiences with prenatal chromosomal microarray analysis and whole-exome sequencing. Orvosi Hetilap 2024, 165(14), 523-530.

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22.11.2025.

Appendix No. 2.

Act LXXIX of 1992 on the Protection of Fetal Life

The Parliament, aware that –

– the life of the fetus, beginning at conception, deserves respect and protection;

– the protection of fetal life can be achieved through increased care for expecting mothers, while at the same time it is primarily the responsibility of the parents to create conditions that ensure the healthy development of the fetus;– that termination of pregnancy is not a means of family planning or birth control;– that family planning is the right and responsibility of parentsenacts the following law:

§ 1 The fetus, which is created from the union of female and male gametes and develops in the womb, and the woman expecting a child are entitled to support and protection.

Means and methods of support and protection

§ 2 (1) Education on the value of health and human life, healthy lifestyles, responsible relationships, dignified family life, and birth control methods that are not harmful to health shall be provided in primary and secondary educational institutions.

(2) The State Family Protection Service or the family protection service registered with the state health administration shall provide counseling, assist in resolving crisis situations, and organize the dissemination of information on family planning outside educational institutions.

(3) The state

a) promotes the use of contraceptive preparations and devices at a reduced price depending on need, the publication of materials promoting the protection of fetal life and explaining birth control, and the dissemination of such information through mass media;

b) promotes the development of a crisis management counseling system that is accessible to mothers and families as a whole and has the appropriate professional expertise, and regulates the conditions and forms of effective cooperation between the state and civil society organizations in the course of counseling;

c) supports activities and organizations that protect fetal life, especially those that provide financial support to pregnant mothers in need;

d) ensures increased protection for pregnant mothers in the workplace through labor law regulations;

e) and the local government shall assist the pregnant mother and her family in bearing and raising the unborn child by providing child welfare and child protection services.

§ 3 (1) The following persons are entitled to free prenatal care

a) Hungarian citizens residing in Hungary,

b) persons who have the right to free movement and residence in accordance with the Act on the Entry and Residence of Persons with the Right to Free Movement and Residence, provided that they have a registered place of residence in accordance with the Act on the Registration of Personal Data and Address of Citizens, and

c) persons subject to the Act on the general rules governing the entry and residence of third-country nationals who have the right of long-term residence.

(2) As part of prenatal care

a) women expecting a child shall be informed about the lifestyle necessary for the healthy development of the fetus, proper nutrition, and the importance of avoiding factors harmful to the fetus (in particular smoking and alcohol consumption);

b) screening tests shall be carried out to monitor the healthy development of the fetus and to ensure the health protection of the pregnant woman;

c) assistance shall be provided to the pregnant woman in preparing for childbirth, breastfeeding, and infant and child care.

§ 4 Termination of pregnancy

§ 5 (1) Pregnancy may only be terminated in cases of danger or serious crisis for the pregnant woman, under the conditions specified in this Act.

(2) A serious crisis is one that causes physical or mental distress or social incapacitation.

§ 6 (1) Pregnancy may be terminated up to the 12th week if

a) it is justified by a reason that seriously endangers the health of the pregnant woman;

b) the fetus is medically likely to suffer from a serious disability or other impairment;

c) the pregnancy is the result of a criminal offense, and

d) the pregnant woman is in a serious crisis situation.

(2) Under the conditions set out in paragraph (1), a pregnancy may be terminated up to the 18th week if the pregnant woman

a) has limited or no legal capacity;

b) is unaware of her pregnancy due to health reasons not attributable to her or due to medical error, or if her pregnancy has exceeded the period specified in paragraph (1) due to the negligence of a healthcare institution or an authority.

(3) Pregnancy may be terminated up to the 20th week – or up to the 24th week in the event of a prolonged diagnostic procedure – if the probability of genetic or teratological damage to the fetus reaches 50%.

(4) Pregnancy may be terminated regardless of its durationa) for h

*eal*th reasons that endanger the life of the pregnant woman, orb) if th

*e f*etus has a disorder that is incompatible with life after birth.

§ 7 (1) Termination of pregnancy, if not justified by health reasons, may be performed on the basis of a written request from the pregnant woman.

(2) In addition to the persons specified in Section 3 (1), a foreign national may also request the termination of pregnancy

a) if she has been residing in the country for more than two months with a valid residence permit,

b) if she has applied for refugee status,

c) who has been recognized as a refugee or an accepted person by the refugee authority, and

d) who, on the basis of an international treaty, cannot be expelled from the country or returned there in accordance with a separate law.

§ 8 (1) A pregnant woman shall submit her request for termination of pregnancy in person to a family protection service employee (hereinafter: employee), together with a certificate issued by an obstetrician-gynecologist confirming the pregnancy.

(2) For the statement of a person with limited legal capacity to be valid, a statement acknowledging the request for termination of pregnancy by their legal representative is required.

(3) The request for termination of pregnancy of a person without legal capacity shall be submitted on their behalf by their legal representative.

§ 9 (1) After the request for termination of pregnancy has been submitted, the staff member shall, if possible in the presence of the father of the fetus, with respect for the feelings and dignity of the pregnant woman, inform her or, in the case specified in Section 8 (3), her legal representative, in order to encourage her to keep the fetus, about

a) the possibility of state and non-state financial and in-kind support available in the event of having the child;

b) the existence and activities of organizations and institutions that provide moral and financial assistance in the event of having the child;

c) the possibilities and conditions of adoption;

d) forms of state, local government or social assistance available to resolve the crisis situation, and offers to assist in accessing these, while at the same time providing the child with information on the possibility of placement in an incubator at a health care institution under the conditions specified in a separate legal regulation, the possibility of placement with the intention of consenting to adoption;

e) conception, fetal development, the risks of termination of pregnancy and its possible effects on future pregnancies;

f) the need to attend family counseling again if the intention to terminate the pregnancy is maintained, at the earliest on the third day after receiving the information specified in points *a) to e)*.

(2) If, despite the information provided in paragraph (1), the applicant still wants to go through with the termination, the staff member will tell them, except in the case mentioned in paragraph (7), at the earliest after the time period mentioned in paragraph (1) point *f)*, about

a) the legal conditions for termination of pregnancy;

b) the circumstances and methods of termination of pregnancy;

c) the health care institutions performing termination of pregnancy; and

d) the possibility of assistance from family protection services after the termination of pregnancy, and at the same time offer assistance in order to ensure appropriate family planning, by providing information on methods of contraception that can be recommended on an individual basis;

e) the possibility of obtaining contraceptive devices at a reduced price.

(3) After providing the information specified in paragraph (2), the staff member shall record the request for termination of pregnancy in writing. The request shall be signed by the applicant and, if possible, by the father of the fetus, and shall specify the health care facility chosen to perform the procedure.

(4) The employee countersigns the request and returns it to the applicant.

(5) The staff member shall send a copy of the countersigned written request to the chosen healthcare institution within 24 hours of handing it over to the applicant.

(6) Persons acting as staff members are bound by confidentiality.

(7) If the pregnancy is the result of a criminal offense, the provisions of paragraph (1) concerning the content of the counseling, the mandatory waiting period following the counseling, and the repeat appearance shall not apply prior to the submission of the request for termination of pregnancy. In this case, the applicant shall also be informed of the possibilities and conditions of adoption.

(8) During the counseling session, until the application for termination of pregnancy is submitted, the pregnant woman shall not be obliged to provide her personal identification data in a manner that would allow her identity to be disclosed.

§ 10 (1) The pregnant woman shall report to the chosen healthcare institution with the application form no later than 8 days after its countersigning. The healthcare institution shall inform the employee who countersigned the application of the termination of pregnancy within 8 days of the procedure.

(3) The pregnant woman shall reconfirm her request by signing it on the day of the procedure.

(4) If the pregnant woman does not report within 8 days, the healthcare institution shall notify the employee by returning a copy of the request form.

(5) If the specialist at the institution performing the procedure determines that the pregnancy has exceeded the time limit specified in this Act or that the procedure would seriously endanger the woman's health, he or she shall refuse to perform the procedure. In this case, the pregnant woman may request a professional review. The pregnant woman shall be informed of the possibility of a review and of the bodies conducting it.

(6) The circle of persons authorized to conduct professional reviews shall be regulated by a ministerial decree.

(7) The procedure approved during the review shall be performed at the health care institution conducting the review.

§ 11 (1) If the pregnant woman does not appear for the professional review within 10 days of refusing the procedure, or if the procedure is definitively refused during the review process, the professional review shall return a copy of the request form to the staff member, who shall immediately notify the public health nurse responsible for the applicant's place of residence.

(2) A pregnant woman

a) whose termination of pregnancy has been definitively refused by the health care institution, or

b) who did not appear for the professional review

shall be taken into care as a high-risk pregnancy.

§ 12 (1) The health reasons justifying the termination of pregnancy in the case of a pregnant woman shall be determined by the unanimous opinion of two specialists who are professionally competent in this field.

(2) The health reasons relating to the fetus shall be determined by the unanimous opinion of two specialists from among the obstetrics and gynecology departments of the genetic counseling center, the prenatal diagnostic center, or the hospital designated by the professionally competent national institute.

(3) The minister shall regulate by decree the circle of persons entitled to the professional review required in the event of a difference of opinion.

(4) The health reasons specified in paragraphs (1) and (2) shall be determined on the basis of the methodological guidelines of the national professional institute or college.

(5) If the pregnancy is the result of a criminal offense, the commission of the offense or the suspicion thereof shall be certified by a certificate issued by the authority conducting the criminal proceedings.

(6) The existence of a serious crisis situation shall be certified by the pregnant woman or, in the event of her incapacity, by her legal representative, by signing the application. In the event of the pregnant woman's incapacity, she shall be given the opportunity to express her opinion on the termination of pregnancy in the proceedings of the family protection service.

Institutions performing abortions

§ 13 (1) Abortions may only be performed in healthcare institutions that meet the conditions set out in the relevant legislation.

(2) State and municipal institutions operating obstetrics and gynecology departments shall ensure the operation of at least one abortion team.

(3) The minister shall determine by decree the health care institutions in which pregnancies exceeding 12 weeks may be terminated.

§ 14 Doctors and health care professionals may not be compelled to perform or assist in the termination of a pregnancy, except in cases where the life of the pregnant woman is at risk.

§ 15 It is prohibited to encourage or promote the termination of pregnancy by any means.

§ 16 (1) The costs of the procedure shall be covered by the Health Insurance Fund if the termination of pregnancy is performed for health reasons relating to the pregnant woman or her fetus who is covered by insurance.

(2) In cases not mentioned in paragraph (1), the fee payable for the termination of pregnancy shall be equal to the current amount of social security financing. The detailed rules for the payment of the fee, including the extent of discounts depending on social circumstances, shall be laid down by the minister in a decree.

(3) The Health Insurance Fund shall advance the amount of the termination of pregnancy financed by social insurance to the health care institution performing the termination of pregnancy. The part of the advanced amount not covered by the fee to be paid shall be reimbursed to the Health Insurance Fund from the budget.

§ 16/A (1) The Government shall be authorized to designate the state family protection service in a decree.

(2) The Government shall be authorized to lay down detailed rules for the registration of the family protection service in a decree.

(3) The minister responsible for health insurance shall be authorized to lay down the conditions for the discounted use of contraceptives and contraceptive products depending on need in a decree.

(4) The minister responsible for health shall be authorized to lay down the detailed rules for prenatal care in a decree.

§ 17 (1) This Act shall enter into force on January 1, 1993.

Compliance with European Union law

§ 18 This Act serves to ensure partial compliance with the following EU legal acts:

a) Council Directive 2003/109/EC of November 25, 2003, concerning the status of third-country nationals who are long-term residents, Article 11(1)(*d)* and Article 21;

(b) Directive 2004/38/EC of the European Parliament and of the Council (29 April 2004) on the right of citizens of the Union and their family members to move and reside freely within the territory of the Member States and amending Regulation (EEC) No 1612/68 and repealing Directives 64/221/EEC, 68/360/EEC, 72/194/EEC, 73/148/EEC, 75/34/EEC, 75/35/EEC, 90/364/EEC, 90/365/EEC and 93/96/EEC, Article 24.